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Original Research

Mite Burden and Immunophenotypic Response to *Demodex musculi* in Swiss Webster, BALB/c, C57BL/6, and NSG Mice

Mariya G Morris,[1*,†] Rodolfo J Ricart Arbona,[1, 2] Kathleen Daniels,[3] Rui Gardner,[3] Imaani Easthausen,[4] William L Boteler,[5] Gregory P Baseler,[5] Gabrielle Pastenkos,[2] Cheryl L Perkins,[6] Kenneth S Henderson,[6] Andrea Schietinger,[7] and Neil S Lipman[1, 2]

Detection methods for *Demodex musculi* were historically unreliable, and testing was rarely performed because its prevalence in laboratory mice was underestimated. Although infestations are unapparent in most mouse strains, *D. musculi* burdens are higher and clinical signs detected in various immunodeficient strains. The parasite's influence on the immune system of immunocompetent mice is unknown. We characterized mite burden (immunocompetent and immunodeficient strains) and immunologic changes (immunocompetent strains only) in naïve Swiss Webster (SW; outbred), C57BL/6NCrl (B6; Th1 responder), BALB/cAnNCrl (BALB/c; Th2 responder) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG; immunodeficient) mice after exposure to *Demodex*-infested NSG mice. Infested and uninfested age-matched mice of each strain (n = 5) were euthanized 14, 28, 56, and 112 d after exposure. Mite burden was determined through PCR analysis and skin histopathology; B-cell and CD4$^+$ and CD8$^+$ T-cell counts and activation states (CD25 and CD69) were evaluated by using flow cytometry; CBC counts were performed; and serum IgE levels were measured by ELISA. Mite burden and PCR copy number correlated in NSG mice, which had the highest mite burden, but not in immunocompetent strains. Infested immunocompetent animals developed diffuse alopecia by day 112, and both BALB/c and C57BL/6 mice had significantly increased IgE levels. These findings aligned with the skewed Th1 or Th2 immunophenotype of each strain. BALB/c mice mounted the most effective host response, resulting in the lowest mite burden of all immunocompetent strains at 112 d after infestation without treatment. Clinically significant hematologic abnormalities were absent and immunophenotype was unaltered in immunocompetent animals. Topical treatment with imidacloprid–moxidectin (weekly for 8 wk) was effective at eradicating mites by early as 7 d after treatment. IgE levels decreased substantially in infested BALB/c mice after treatment. These findings demonstrate a need for *D. musculi* surveillance in mouse colonies, because the infestation may influence the use of infested mice in select studies.

Abbreviations: dpi, days after infestation; dpt, days after treatment; TG, treatment group

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Demodex spp. have evolved over millions of years as host-specific microfauna to reside in low numbers within hair follicles or sebaceous glands. The mites infest almost all mammalian species and are generally host-specific.[46] Although most hosts are typically asymptomatic, immunosuppression may increase mite numbers and clinical signs, such as alopecia, erythema, pruritus, and crusting. Currently, 5 *Demodex* spp. have been identified in mice,[5-7,40] but naturally occurring infestations in laboratory mice are rare and have only been attributed to *D. musculi*.[2] This finding is not surprising, given that most laboratory mouse strains have been rederived by cesarean section early in their development, and the mites are transmitted horizontally. The production, sharing, and importation of genetically engineered strains may have led to the inadvertent introduction of *D. musculi* into many colonies. This occurred because until recently, detection methods were unreliable, and testing for the parasite was not routinely performed as its presence in laboratory mice was mostly unrecognized or ignored. A prevalence of approximately 10% is estimated in academic colonies based on PCR data from animals imported into Memorial Sloan Kettering Cancer Center and Weill Cornell Medicine from other institutions.[43]

Although infestations are subclinical in most mouse strains, *D. musculi* burdens are higher and clinical signs are reported in several strains of immunodeficient mice, including SCID, TRP1/TCR, strains lacking both CD28 and STAT6, and transgenic animals that overexpress *CD3E* or *Prad*.[18,30,37,51] Downregulation of Th2 immunity predisposes mice to infestation with *D. musculi*.[51] Although the immunophenotypes of the mice in these reports were known and likely the cause of increased susceptibility, the

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[1]Tri-Institutional Training Program in Laboratory Animal Medicine and Science, Memorial Sloan Kettering Cancer Center, The Rockefeller University, and Weill Cornell Medicine, New York, New York; [2]Center of Comparative Medicine and Pathology, Memorial Sloan Kettering Cancer Center and Weill Cornell Medicine, New York, New York; [3]Flow Cytometry Core Facility, Memorial Sloan Kettering Cancer Center, New York, New York; [4]Division of Biostatistics and Epidemiology, Department of Healthcare Policy and Research, Weill Cornell Medicine, New York, New York; [5]XPressBio, Frederick, Maryland; [6]Research Animal Diagnostic Services, Charles River Laboratories, Wilmington, Massachusetts; [7]Program in Immunology, Memorial Sloan Kettering Cancer Center, New York, New York
[*]Corresponding author. Email: mgm2195@cumc.columbia.edu
[†]Current affiliation: Institute of Comparative Medicine, Columbia University Irving Medical Center, New York, New York

effect (if any) of *D. musculi* on the immune system of immunocompetent mice remains to be determined.

A familiar veterinary demodecid mite, *D. canis*, has long been known to cause a spectrum of disease in dogs, and most studies conducted in this species focus on immunologic defects[16] that lead to the progression of clinical signs.[32] The mite may cause localized immunosuppression, evidenced by the ability of the host to tolerate small numbers of mites normally.[9,16,48] Several theories have been proposed regarding the immunologic pathology, including immunosuppression of T lymphocytes by mites, altered $CD4^+$:$CD8^+$ T-cell ratios and activity, and upregulation of immunosuppressive cytokines, as well as hematologic abnormalities, including anemia.[27,28,50] Dogs with generalized demodicosis have increased TGF mRNA, TGFβ (a potent immunosuppressor), and decreased TNFα.[15,48-50,52] These changes demonstrate that *D. canis* interacts with the host immune system in a multitude of ways; similar changes may occur with *D. musculi* and could have important undesired effects on mice inadvertently infested with *D. musculi* used as research models.

Historically, *D. musculi* has been identified via fur pluck, deep skin scrape, and histopathology—all of which are time-consuming and have a high incidence of false-negative results in the face of low or moderate mite burdens. Testing of imported animals for pathogens ideally should be noninvasive. Therefore, PCR analysis is the ideal method for identifying *D. musculi* in laboratory mice. PCR testing, which has become commercially available recently, has been confirmed to be a valuable diagnostic tool for *D. musculi*[37,39] as well as other murine ectoparasites.[26]

Clinically inapparent parasitic infections in animal models can have immunologic effects, thus altering research results. As examples, the murine intestinal protozoa *Tritrichomonas muris* upregulates Th1 mucosal immunity,[8,12,13] and fur mite infestations in NC/KuJ, C57BL/6, and BALB/c mice increase IgE levels.[29,35,41,44] lnfestation with the fur mite *Myocoptes musculinus* results in increased Th2 cytokine production in C57BL/6[34] and BALB/c mice,[23] produces long-standing pathologic changes even after eradication,[22] and increases susceptibility to *Toxoplasma gondii* in mice that are otherwise resistant.[56] Similar findings are obtained in mice infected with helminths.[31] Furthermore, whether the treatment modality of choice for *D. musculi*—a commercially available imidacloprid–moxidectin product—has any effect on immunologic parameters has not yet been established.[38] In some studies, the administration of the anthelminthic fenbendazole alters the onset of autoimmune encephalomyelitis[14] but does not alter broad immunologic parameters in BALB/c mice.[12] It is plausible that similar changes may be seen with *D. musculi* infestation, and it is important to determine whether immunologic effects (if they occur) normalize after mite eradication.

We hypothesized that *D. musculi* infestation, even in the absence of clinical signs, would alter quantitative or qualitative immunologic parameters in 3 commonly used immunocompetent mouse strains. Given that Th2-mediated immune responses are known to increase resistance to extracellular parasitic infections,[19,45] the Th1-skewed C57BL/6 strain might sustain a higher mite burden than the Th2-skewed BALB/c[47] strain or the heterogeneous-responding Swiss Webster (SW) stock. We also sought to determine whether the host's immune parameters—if altered as a result of infestation—returned to baseline after treatment and mite eradication. Finally, because PCR testing has become the preferred diagnostic technique for detecting infestation, we evaluated whether *D. musculi* PCR copy number correlated with the number of mites present in hair follicles.

Materials and Methods

Experimental animals. Crl:CFW(SW) (SW, n = 40), C56BL/6NCrl (B6, n = 40), and BALB/cAnNCrl (BALB/c, n = 65); Charles River Laboratories [CRL], Wilmington, MA) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ ([NSG], n = 40; obtained from a breeding colony at Memorial Sloan Kettering Cancer Center [MSK]) mice (age, 4 to 6 wk) were used. Three B6.Cg-*Rag1*$^{tm-1Mom}$*Tyrp1*$^{B-wTg}$(Tcra,Tcrb)9Rest/M (TRP1/TCR; TRP) mice from a *D. musculi*-positive colony served as a source of *D. musculi* to infest NSG mice that were subsequently used to infest the other strains. All mice were housed in solid-bottom, polysulfone, shoebox-style, IVC (Maxi-Miser, Thoren Caging Systems, Hazelton, PA). Each cage contained autoclaved aspen chip bedding (PWI Industries Canada, Quebec, Canada); 2 pieces of steam-sterilized, compressed, cotton nesting material (0.5 in^2; Cotton squares, Ancare, Bellmore, NY); flash-autoclaved, γ-irradiated feed[54] with or without 0.12% amoxicillin (LabDiet 5053, PMI, St Louis, MO); and acidified water (pH 2.5 to 2.8) provided ad libitum in a polysulfone bottle with a neoprene stopper (Thoren Caging Systems). The holding room was maintained on a 12:12-h light:dark cycle with room temperature of 72 ± 2 °F (22.2 ± 0.5 °C) and relative humidity ranging from 30% to 70%. The studies conducted were approved by Memorial Sloan Kettering's IACUC. The animal care and use program is AAALAC-accredited.

Mice were free of the following agents: mouse hepatitis virus, Sendai virus, mouse parvoviruses, minute virus of mice, pneumonia virus of mice, Theiler meningoencephalitis virus, epizootic diarrhea of infant mice (mouse rotavirus), ectromelia virus, reovirus type 3, lymphocytic choriomeningitis virus, K virus, mouse adenovirus 1 and 2, polyoma virus, murine cytomegalovirus, mouse thymic virus, Hantaan virus, *Mycoplasma pulmonis*, *Citrobacter rodentium*, *Salmonella spp.*, ciliary-associated respiratory bacillus, and *Clostridium piliforme*; fur mites (*Myobia musculi*, *Myocoptes musculinis* and *Radfordia affinis*), pinworms (*Syphacia spp.* and *Aspiculuris spp.*), and *Encephalitozoon cuniculi*. NSG mice used as *Demodex* donors were also free of *Spironucleus muris*, *Giardia muris*, *Entamoeba muris*, and *Tritrichomonas muris*.

Infestation. Cohousing 12 female NSG mice with 3 male *Demodex*-infested TRP mice (4 NSG:1 TRP per cage) for a minimum of 14 d created a colony of "Demodex-donor" NSG mice. Animals were confirmed positive via fur pluck, deep skin scrape, and/or PCR, and these mice were then cohoused with an additional 48 NSG female mice to expand the infested colony to a total of 60 infested NSG mice. NSG mice were provided an amoxicillin-compounded diet to minimize opportunistic infection and normalize skin flora until cohoused with immunocompetent strains.

Experimental animals were housed in cages of 2 to 4 experimental animals with a single NSG *Demodex*-donor or *Demodex*-negative mouse. Prior to cohousing, all experimental animals and *Demodex*-negative NSG mice were confirmed to be negative for *D. musculi* via PCR analysis of pooled samples. Mice were cohoused until they were euthanized. Mice were selected randomly, and the remaining animals were rehoused to generate the fewest number of cages.

Study design. A *Demodex*-positive (confirmed via fur pluck or deep skin scape) or *Demodex*-negative NSG (confirmed via PCR analysis of pooled samples) mouse was cohoused in the same cage with 4 naïve SW, C57BL/6, BALB/c, or NSG mice (n = 20 per stock or strain per group [infested and noninfested]) for 14, 28, 56, or 112 d. At each time point, 5 mice from each group were randomly selected, evaluated for skin lesions and behavioral changes, euthanized with carbon dioxide, and skin swabs for

PCR, blood for CBC and IgE determination (except NSG mice), pelt for histopathology and mite burden determination, and spleen for immunophenotyping (except NSG mice) were collected and analyzed. The time points were selected according to the life cycle of *D. canis*, which is 18 to 24 d,[31] given that the life cycle of *D. musculi* is unknown but is likely similar.

To determine whether various immunologic parameters return to baseline after treatment, a cohort of infested BALB/c mice (n = 25) was created by cohousing 4 naïve BALB/c mice with a confirmed-infested NSG mouse for 42 d. After cohousing, 5 animals were selected randomly and euthanized, and samples were collected for PCR and serum IgE analysis. The remaining mice were randomly divided into 2 groups (treatment group [TG] and controls; n = 10 each group) after the NSG mouse was removed. An additional group, TG 2, consisting of 5 *Demodex*-negative (confirmed via PCR analysis of pooled samples) BALB/c mice were housed separately. All animals were treated with miticide or vehicle once weekly for 8 wk. After completion of the treatment course, 5 animals from TG 1 and controls were randomly selected and euthanized, and skin swabs for PCR analysis, blood for CBC and IgE determination, and pelt were at collected 7 and 28 d after treatment (dpt). The 5 mice from TG 2 were anesthetized with isoflurane (1% to 4%), and approximately 100 µL of blood was collected via the retroorbital sinus for serum IgE concentration at the same time points.

PCR analysis. Immediately after euthanasia and blood collection, a pelt swab was collected for PCR by moving an adhesive swab (Pigeon Corporation, Tokyo, Japan) against the direction of fur growth along the entire length of the dorsum, ventrum, and each flank, while applying gentle pressure and rotating the swab as it moved. Antemortem swab collection was conducted under gentle manual restraint. Samples from infested animals were assayed individually, whereas samples from uninfested animals were pooled in groups (n = 5). The areas swabbed mirrored the areas examined microscopically. Swabs were stored in 1.5-mL conical microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) at –80 °C until tested en masse.

PCR analysis was performed on lysates obtained from adhesive swabs, by using a proprietary real-time fluorogenic 5′ nuclease TaqMan PCR assays targeting conserved regions of 18S rRNA (Charles River Research Animal Diagnostic Services, Wilmington, MA). The assay is broadly reactive for multiple species of *Demodex*, including *D. musculi*.

Copy number was estimated for any sample demonstrating amplification. The cycle at which the test sample crossed the fluorescence threshold (C_t) was compared with the C_t of the plasmid-based positive template control, which was calculated to represent 100 target copies. A simple calculation was performed where every 3.3 cycles represents 1 log10 difference in copy number.

Pelt harvest and analysis. After collection of the pelt swab, 4 approximately 1-cm–wide sections of skin were removed from the length of the dorsum (tail base to skull base), the length of the ventrum (pelvis to mandible), and from the inguinal to axillary folds of each of the flanks. Each section of skin was laid flat on a piece of cardboard and stored in 10% neutral buffered formalin. After a minimum of 48 h of fixation, 2 3- to 5-mm–wide sections were trimmed from the cranial and caudal aspects of each strip of skin, paraffin-embedded, and stained with hematoxylin and eosin. Each section was identified by using tissue marking dye to signify the location. The length of the 8 skin sections was measured by using image analysis software (CellSens Dimension, Olympus, Center Valley, PA), and the number of mites in hair follicles was counted by using light microscopy. The total number of mites divided by the sum of the 8 skin sections yielded the number of mites per centimeter of skin. Cranial and caudal aspects of dorsal and ventral skin sections from uninfested (n = 1 per time point) and infested (n = 5 per time point) mice at 56 and 112 d after infestation (dpi) and 7 and 28 dpt TG 1 (n = 5 per time point, infested, miticide treated) and control (n = 5 per time point, infested, vehicle treated) BALB/c mice were also evaluated by a veterinary pathologist (GP) for evidence of acanthosis, hyperkeratosis, and dermatitis.

Hematology. Immediately after euthanasia, blood was collected into either a Ca-EDTA or a serum separator tube (BD Microtainers, Becton Dickinson, Franklin Lakes, NJ) via terminal cardiocentesis for CBC or IgE determination. Samples were refrigerated for a maximum of 2 h prior to processing. The serum separator tube was centrifuged at 4696 × g for 10 min, and the serum was separated and stored in a 1.5-mL conical microcentrifuge tube at –80 °C until analyzed. CBC counts were performed by using an automated analyzer (ProCyte Dx Hematology Analyzer, IDEXX Laboratories, Westbrook, ME). Parameters evaluated included RBC count, Hgb concentration, Hct, reticulocyte count, WBC count, neutrophil count, lymphocyte count, basophil count, and eosinophil count, as well as percentages of all populations.

Immunophenotyping. After the pelt was harvested, the spleen was removed for flow cytometry. The spleen was dissociated into a single-cell suspension, and RBC were lysed by using ACK lysis buffer (catalog no. TNB-4300, Tonbo Biosciences, San Diego, CA). The suspension was washed twice with PBS, and then stained for flow cytometry. Cells were blocked with Fc Receptor Block (catalog no. 101302, BioLegend, Dedham, MA) prior to staining. Spleens were then stained with the following antimouse antibodies: CD8a-FITC (53-6.7, catalog no. 100706), CD69-BV 421 (H1.2F3, catalog no. 104527), NK1.1-PerCP/Cy5.5 (PK136, catalog no. 108728), CD25-PE (PC61, catalog no. 102008), F4/80-PE/Cy5 (BM8, catalog no. 123112), Ly-6C-PE/Cy7 (HK1.4, catalog no. 128018), CD4-APC (GK1.5, catalog no. 100412), CD45R/B220-APC/Cy7 (RA3-6B2, catalog no. 103224), Ly-6G-BV510 (1A8, catalog no. 127633), and CD11b-BV605 (MI/70, catalog no. 101257; all from BioLegend) and CD45.2-AF700 (104, catalog no. 56-0454-82), CD19-PE-TxRed (6D5, catalog no. RM7717), and 4′,6-diamidino-2-phenylindole (DAPI) for discrimination of live and dead cells (all from ThermoFisher, Waltham, MA). Super Bright staining buffer (catalog no. SB-4400-42, ThermoFisher) was added to staining cocktails. Fluorescence-minus-one controls for the following antibody–fluorophore conjugates were created to validate gating: NK1.1-PerCP/Cy5.5, CD25-PE, F4/80-PE/Cy5, Ly-6c-PE/Cy7, Ly-6G-BV510, CD11b-BV605, and CD45.2-AF700. Samples were acquired by using an Aurora Spectral Analyzer (Cytek Biosciences, Fremont, CA) equipped with 3 lasers with full spectral detection from 421 to 489 nm and 498 to 829 nm with the 405-nm laser, 498 to 630 nm and 652 to 829 nm with the 488-nm laser, and 652 to 829 nm with the 640-nm laser. Data were analyzed by using FlowJo version 10.5.0 (BD Life Sciences – Informatics, Ashland, OR). Population percentages were calculated for B cells, $CD4^+$ cells, $CD8^+$ cells, neutrophils, and monocytes (Figure 1). Stain index was calculated for activation markers CD25 and CD69 on $CD4^+$ and $CD8^+$ cells by using a previously published formula.[25]

Analysis of total serum IgE. Total IgE concentrations were determined from previously frozen, masked serum samples by using a commercially available total mouse IgE ELISA (Mouse Total IgE ELISA Kit, XpressBio Life Science Products, Frederick, MD) according to instructions from the assay manufacturer.

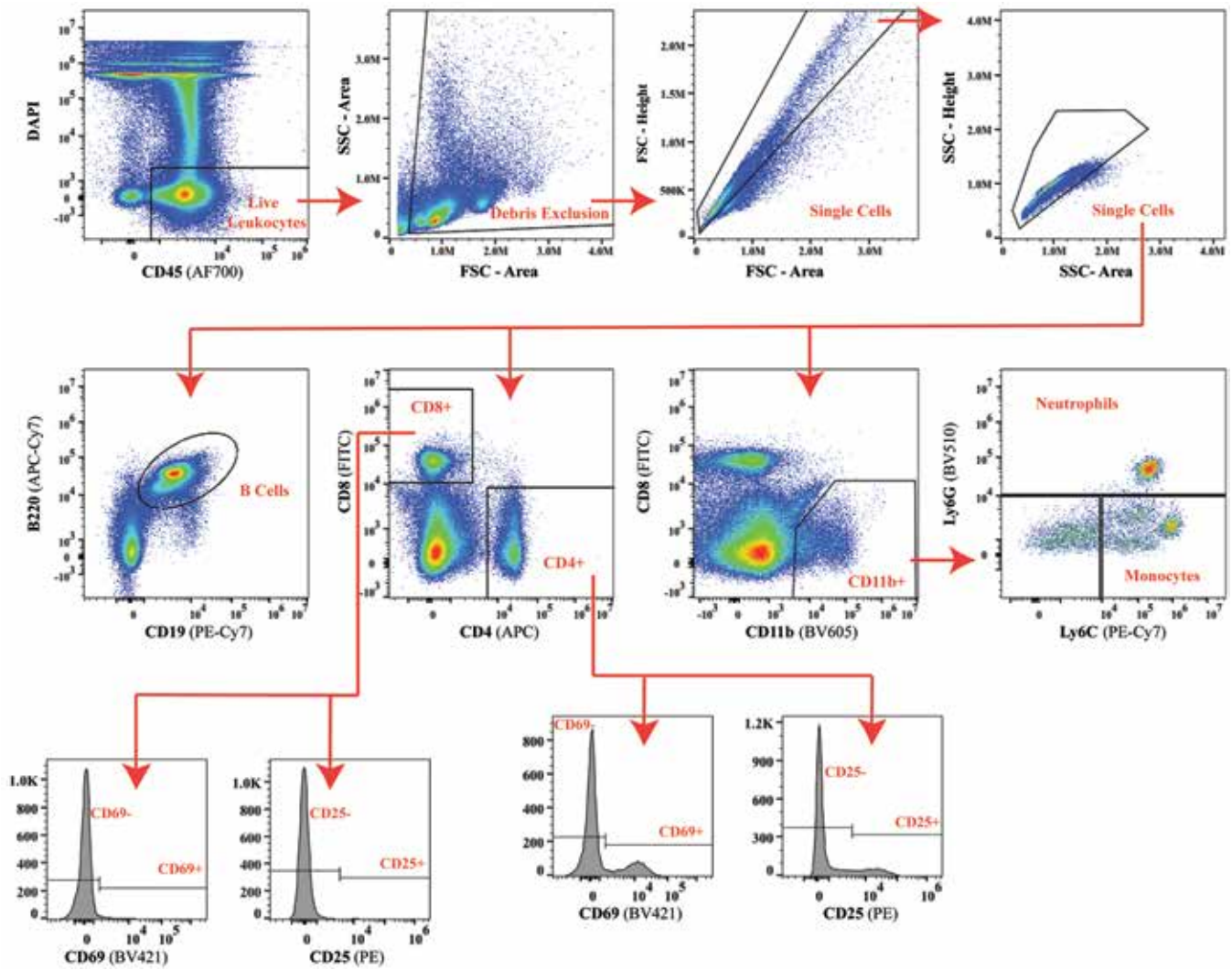

Figure 1. Flow cytometry gating strategy for cell populations of relevance in splenocytes from an uninfested B6 mouse at 56 dpi. Live leukocytes were identified by positive expression of CD45 and negative for the viability dye DAPI, followed by debris removal and doublet discrimination. B cells were quantified as $CD19^+$ and $B220^+$; CD8 and CD4 T cells were identified by expression of their respective marker, whereas neutrophils and monocytes were quantified as $CD11b^+$ and further distinguished as $Ly6G^+$ and $Ly6G^-Ly6C^+$, respectively. CD25 and CD69 activation in $CD4^+$ or $CD8^+$ T cells was determined by using the stain index formula presented previously.[26]

In brief, serum samples were diluted 1:50 in sample diluent, dispensed into microtiter wells coated with antimouse IgE antibody, and incubated at 37 °C for 60 min. Diluent was aspirated and wells washed prior to addition of antimouse IgE conjugated to horseradish peroxidase. Conjugate was removed and wells washed before substrate solution (tetramethylbenzidine) was added. Reaction was stopped by the addition of stop solution (1 N H_2SO_4) and absorbance values read at 450 nm. Samples were run in duplicate, and results from a known standard curve were used to determine total IgE concentration.

Miticide treatment. Mice in TG 1 and 2 were treated with moxidectin–imidacloprid (Advantage Multi, Bayer, Shawnee Mission, KS) as described.[38] The product was diluted in 70% ethanol to achieve a 3% solution. Animals were treated at cage change weekly for 8 wk, with TG 2 animals handled first, followed by TG 1 and controls. All treated animals were weighed individually immediately prior to treatment, to provide a dose of approximately 3 mg/kg moxidectin and 13 mg/kg imidacloprid. Animals received 74 to 94 µL between the scapulae by using a single-channel micropipette (Pipetman Classic P100, Gilson, Middleton, WI). Vehicle-treated mice received 80 µL ethanol between the scapulae per mouse at cage change weekly for 8 wk.

Statistical analysis. The center and spread of each variable of interest was described as median and range for each strain or group at each time point. Meaningful pairwise comparisons were made by using Wilcoxon rank-sum tests. Kruskall–Wallis tests were used to compare 3 or more groups. Spearman correlation tests were used to assess for relationships between mite burden and time for each strain.

All *P* values are 2-sided, and all hypotheses were evaluated at the 0.05-α level. 95% CI were developed on 1000 bootstrap samples to assess the precision of Spearman correlation coefficients. Because of the small sample sizes, correction for multiple hypothesis testing was not performed. Given that the investigators a priori hypothesized negative results for the majority of

Table 1. Mice with evidence of alopecia[a]

	SW		B6		BALB		NSG		All	
Time point	Uninfested	Infested	Uninfested	Infested	Uninfested	Infested	Uninfested	Infested	Uninfested	Infested
14 dpi	0/5	0/5	1/5	3/5	0/5	0/5	0/5	0/5	1/20	3/20
28 dpi	0/5	0/5	1/5	3/5	0/5	4/5	0/5	0/5	1/20	7/20
56 dpi	1/5	3/5	2/5	5/5	0/5	2/5	0/5	0/5	3/20	10/20
112 dpi	3/5	5/5	3/5	5/5	1/5	4/5	0/5	5/5	7/20	19/20
Total	4/20	8/20	7/20	16/20	1/20	10/20	0/20	5/20	12/80	39/80

Data are given as no. of affected mice/total no. of mice in group
[a]Alopecia is defined as any area of visible skin which is normally haired.

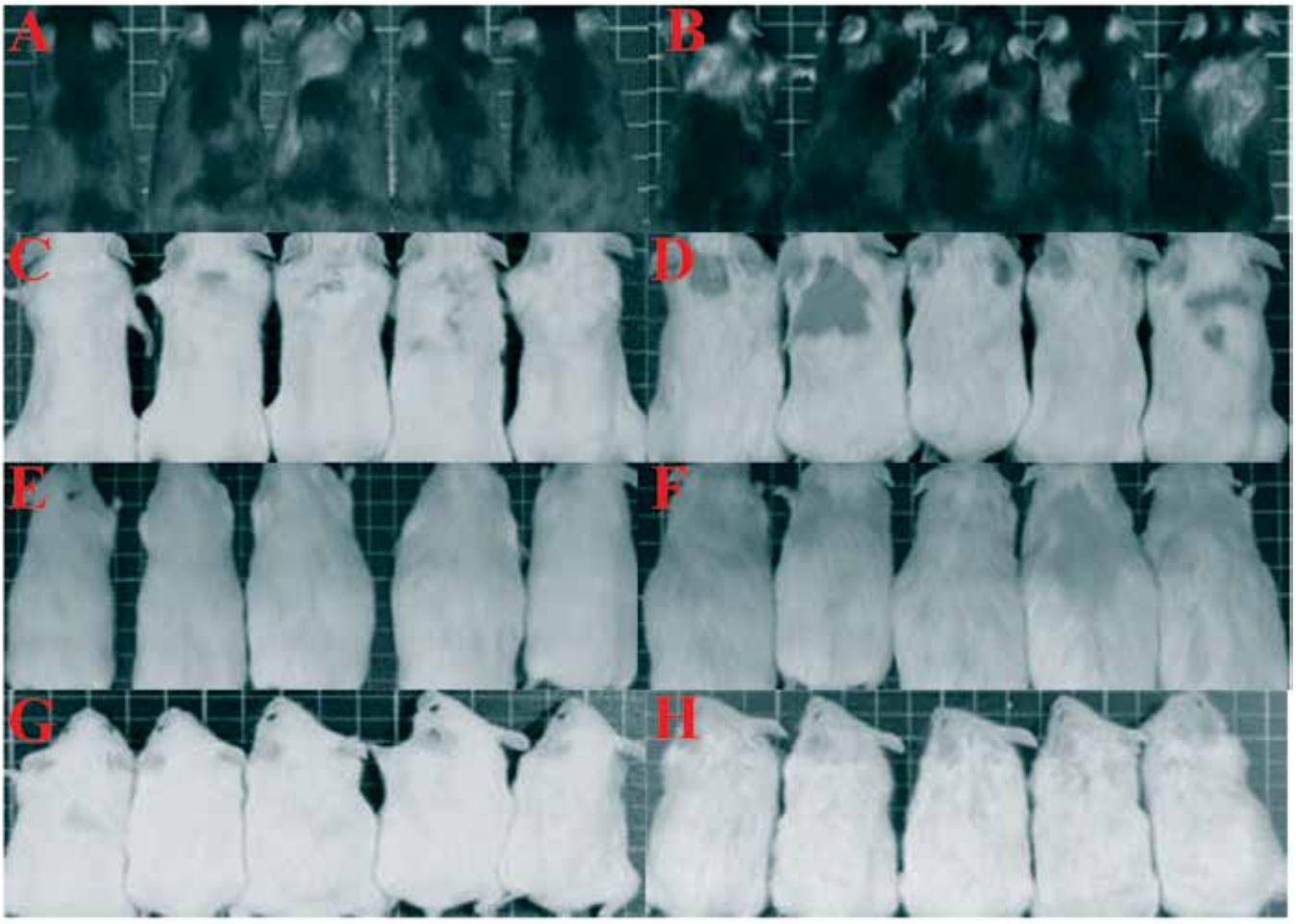

Figure 2. Dorsal pelt view of (A, C, E, G) uninfested and (B, D, F, F) infested (A, B) B6, (C, D) BALB/c, (E, F) SW, and (G, H) NSG mice at 112 dpi. Infested mice (B, D, E, F) have substantial hair loss or alteration in coat texture compared with uninfested mice (A, C, E, G).

tests performed, this approach is conservative. Analyses were performed in R version 3.5.3 (http://www.R-project.org).

Results

Behavioral changes and skin lesions. Infested animals were not pruritic when observed nor did they have evidence of pruritus (erythema, excoriations, or crusting). A large proportion of the immunocompetent infested mice developed alopecia by 112 dpi (Table 1). Photographs of the dorsal pelage of uninfested and infested mice of all strains at 112 dpi are shown in Figure 2. Uninfested B6 and BALB/c mice had lesions consistent with barbering; however, the proportion of mice with lesions and magnitude of fur loss were greater in infested mice. All infested SW mice developed diffuse fur loss over the scapulae and the interscapular region. NSG mice developed ruffled fur on the face and the dorsal cervical region; no alopecia was noted.

Mite burden, PCR findings, and their correlation. Mites were identified microscopically in at least 1 skin section of each mouse exposed to an infested NSG mouse. The number of skin sections without mites ranged from 15% to 75%, depending on strain and time point (Table 2). Mite burden (median and interquartile range) and temporal changes by strain are provided in Figure 3 A. The median mite burden was highest among NSG mice at each of the 4 time points as compared with the immunocompetent strains. Median mite burden was significantly higher

Table 2. Percentage of skin sections of mite-exposed mice (n = 5 mice/strain/time point) without histologic evidence of *D. musculi* (n = 8 skin sections/mouse)

	14 dpi	28 dpi	56 dpi	112 dpi
SW	70.0%	75.0%	75.0%	57.5%
B6	62.5%	50.0%	47.5%	35.0%
BALB/c	47.5%	55.0%	75.0%	65.0%
NSG	15.0%	0.0%	0.0%	0.0%

at 28 dpi ($P_{NSG,SW}$ = 0.01, $P_{NSG,BALB}$ = 0.01, $P_{NSG,B6}$ = 0.01), 56 dpi ($P_{NSG,SW}$ = 0.01, $P_{NSG,BALB}$ = 0.01, $P_{NSG,B6}$ = 0.01), and 112 dpi ($P_{NSG,SW}$ = 0.01, $P_{NSG,BALB}$ = 0.01, $P_{NSG,B6}$ = 0.01). At 14 dpi, NSG mice had significantly higher mite burdens than SW (P = 0.02) and B6 (P = 0.04) mice only (Figure 4). The median mite burden in NSG mice increased from 1.17 mites/cm at 14 dpi to 52.4 mites/cm at 112 dpi (correlation coefficient, 0.97; 95% CI, 0.93 to 0.0.97; P < 0.001). The maximal median mite burden observed among the other strains at any time point was 0.80 mites/cm in B6 mice at 112 dpi. There were no statistically significant differences in mite burden between any of the immunocompetent strains at 14, 28, or 56 dpi. At 112 dpi, B6 had a statistically higher mite burden than BALB/c and SW mice. There was no significant difference in median mite burden between BALB/c and SW mice at 112 dpi.

PCR assay results were positive for all mice at all time points except for a single SW mouse at 14 dpi, but mites were observed in skin sections at all time points. Among the immunocompetent strains, median PCR copy numbers were increased at 56 dpi when compared with 14 dpi and had decreased again at 112 dpi when compared with 56 dpi. Copy numbers for NSG mice increased steadily throughout the study (Figure 3 B). Among NSG mice, higher mite burden correlated strongly with an increased copy number (correlation coefficient, 0.95; 95% CI, 0.82 to 0.98, P < 0.001). Correlations between mite burden and copy number were not observed among the immunocompetent strains (data not shown).

Histopathology. A single uninfested animal was evaluated at both 56 and 112 dpi. No epidermal changes were observed in any of the skin sections; however, both mice had minimal dermal inflammation. At 56 dpi, 4 of 5 infested animals had mild to moderate acanthosis and hyperkeratosis in at least one skin section, and all had a mild to severe dermal inflammation in the majority of sections evaluated. Dermal inflammation was primarily lymphocytic and granulocytic, with mast cell infiltrates in a small number of sections. At 112 dpi, all animals had minimal acanthosis and hyperkeratosis in at least 2 of 4 skin sections and minimal to mild dermal inflammation with a similar inflammatory pattern and distribution as described at 56 dpi.

The frequency and distribution of lesions in infested, untreated animals at 7 dpt was similar to that described in animals at 56 and 112 dpi, yet lesions did not significantly differ from the changes identified in control, vehicle-treated animals. At 7 dpt, only 3 animals had mild to moderate either acanthosis or keratosis or both. All, however, had mild to moderate dermatitis. By 28 dpt, mild acanthosis was present in one skin section from a single animal, 3 mice had mild hyperkeratosis, and 4 animals had mild dermatitis.

Hematology. CBC results are provided in Tables 3 and 4. Only a few statistically significant differences in the leukograms were observed between uninfested and infested mice, and no consistent patterns were observed across cell types over time. Some results were slightly outside of the vendor's strain-, sex-, and age-specific published reference ranges. For

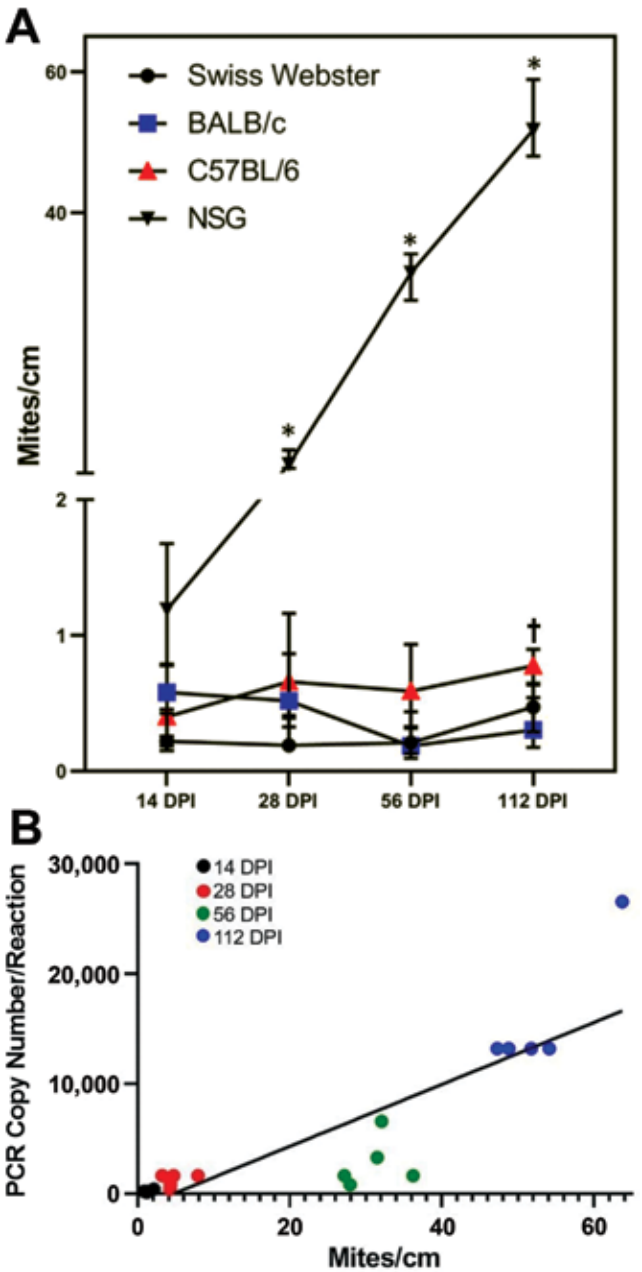

Figure 3. (A) Mite burden (median and interquartile range) by strain (n = 5 per strain per time point) from 14 to 112 dpi. At 14 dpi, NSGs had significantly higher mite burdens than SW (P = 0.02) and B6 (P = 0.04) mice. NSG mice had significantly higher mite burdens than the other 3 strains at 28, 56, and 112 dpi (*, $P \leq 0.05$). B6 mice had a *significantly* higher mite burden than BALB/c mice at 112 dpi (†, $P \leq 0.01$). SW mice demonstrated a moderate increase in median mite burden over time from 0.20 mites/cm at 14 dpi to 0.42 mites/cm at 112 dpi (correlation coefficient, 0.51; 95% CI, 0.02 to 0.79, P = 0.02), there was no trend among B6 mice, and BALB/c mice showed a moderate decreasing trend over time from 0.67 mites/cm at 14 dpi to 0.27 mites/cm at 112 dpi (correlation coefficient, –0.46; 95% CI, –0.80 to –0.01; P = 0.04). There were no statistically significant differences in mite burden between any of the immunocompetent strains at 14, 28, or 56 dpi, but this relationship was unstable at 112 dpi (P = 0.02). At 112 dpi, B6 had a statistically higher mite burden than BALB/c (P = 0.02) and SW (P = 0.03) mice. There was no significant difference in median mite burden between BALB/c and SW mice at 112 dpi. (B) Correlation between mite burden and PCR copy number for NSG mice. All time points are shown; black, 14 dpi; red, 28 dpi; green, 56 dpi; and blue, 112 dpi.

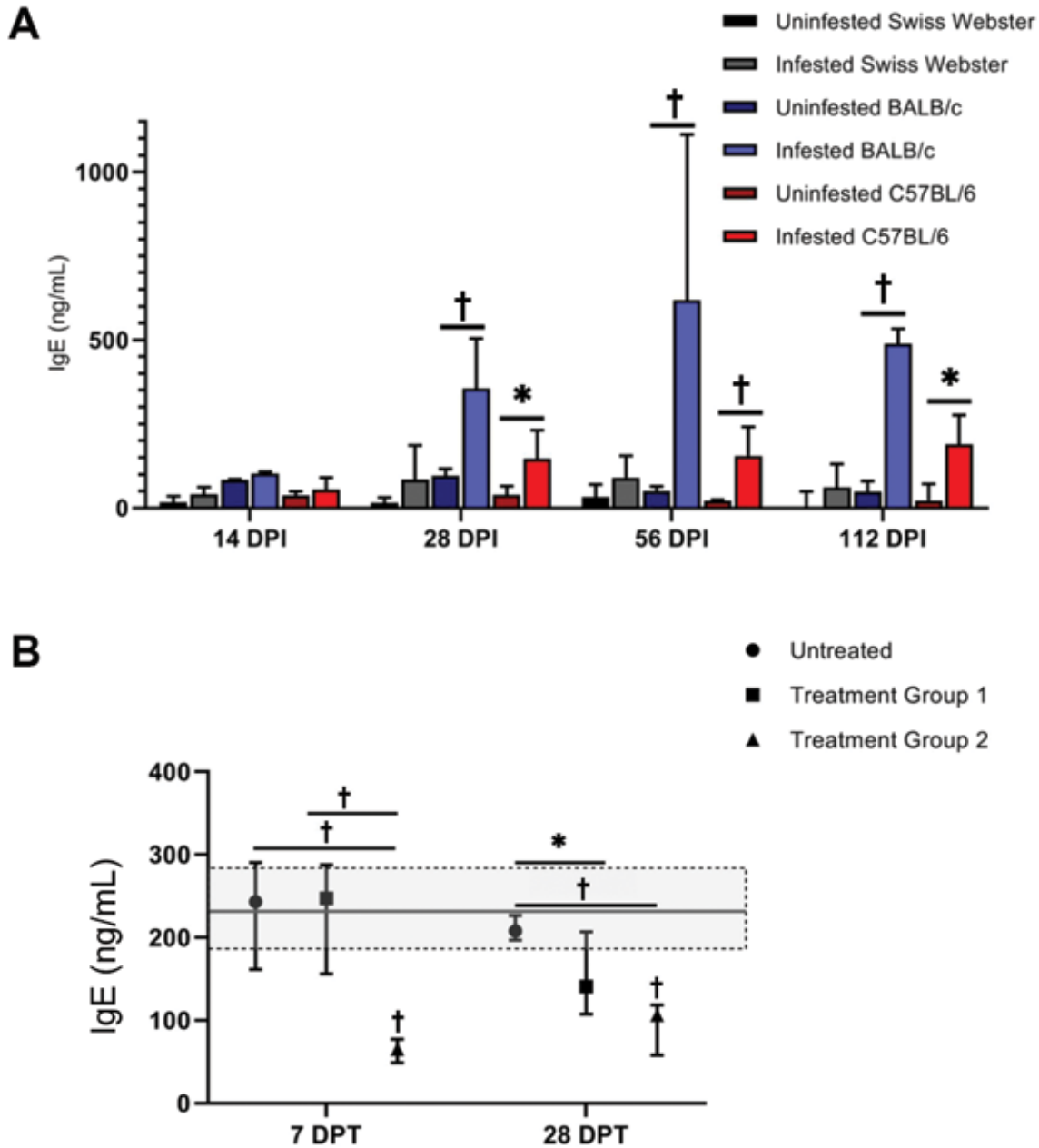

Figure 4. (A) Serum IgE concentration (median and interquartile range [bars]) for infested and uninfested (control) SW, B6, and BALB/c mice at 14, 28, 56, and 112 dpi (n = 5 mice/group/strain/time point). Significant differences between infested and uninfested mice of the same strain are shown (*, $P \leq 0.05$; †, $P \leq 0.01$). (B) Serum IgE concentrations (median and interquartile range [bar]) for control (untreated), treatment group 1 (*Demodex*-positive; treated) and treatment group 2 (*Demodex*-negative; treated) BALB/c mice at 7 and 28 d after treatment (dpt; n = 5 mice/group/time point). Solid and dashed lines indicate the median and interquartile range, respectively, of infested BALB/c mice (n = 5; randomly selected) prior to initiating treatment. Significant differences between groups are shown (*, $P \leq 0.05$; †$P \leq 0.01$). At both 7 and 28 dpt, *Demodex*-negative treatment group 2 mice had significantly lower serum IgE concentrations than *Demodex*-positive mice at 42 dpi.

example, the median lymphocyte count was significantly increased (P = 0.012) in infested as compared with uninfested SW mice at 28 dpi, and the increased values were outside of the upper limit of the published reference range.[11] However, there was no difference for these parameters at the 3 other time points evaluated, and the directions of the differences between infested and uninfested animals were not consistent over time. Infested B6 mice had significantly decreased median WBC and lymphocyte counts (P = 0.012 and P = 0.022, respectively) at 28 dpi. The median WBC count at this time point was below the lower limit of the reference range; however, the median value for the uninfested mice was above the upper limit of the reference range.[10] Infested BALB/c mice only had a significantly higher median neutrophil count as compared with uninfested controls at 112 dpi, and this value was within reference ranges.

Hemogram changes were clinically and biologically unremarkable. Although in several cases the infested mice had significantly different RBC counts or Hct values as compared with the uninfested (control) mice at the same time point, data were within the vendor's published reference ranges, and the observed changes were not consistent between strains or time points.

Table 3. WBC, neutrophil, lymphocyte, and eosinophil counts (median and IQR) for infested and uninfested (control) SW, B6, and BALB/c mice at 14, 28, 56 and 112 d after *Demodex* infestation (dpi; n = 5 mice/group/strain/time point)

	WBC (× 10^3/µL)				Neutrophils (× 10^3/µL)				Lymphocytes (× 10^3/µL)				Eosinophils (× 10^3/µL)			
	Control	IQR	Infested	IQR	Control	IQR	Infested	IQR	Control	IQR	Infested	IQR	Control	IQR	Infested	IQR
SW 14 dpi	4.37	0.08	8.16	4.77	0.49	0.27	0.83	0.28	3.76	0.39	6.25	3.10	0.05	0.01	0.13	0.04
SW 28 dpi	7.63	0.16	12.65	2.38	0.87	0.25	1.26	0.45	6.41	0.22	11.09[b,c]	1.73	0.03	0.03	0.27[a]	0.20
SW 56 dpi	8.53	2.20	10.25	4.17	1.01	0.25	0.86	0.15	7.04	1.78	6.61	2.29	0.10	0.03	0.14	0.06
SW 112 dpi	7.61	1.48	8.65	3.42	1.04	0.41	1.15	0.01	6.11	1.55	7.11	3.57	0.10	0.14	0.16	0.02
B6 14 dpi	6.68	1.73	6.66	1.76	0.60	0.17	0.87	0.67	4.90	2.54	5.02	1.56	0.13	0.01	0.10	0.06
B6 28 dpi	9.65	4.93	5.95[b,d]	1.42	3.54	4.28	1.36	0.74	6.94	0.81	4.69[a]	1.25	0.27	0.08	0.15	0.04
B6 56 dpi	10.29	0.13	9.33	0.19	0.92	0.34	1.34	1.95	8.43	0.55	5.84	0.58	0.22	0.06	0.19	0.06
B6 112 dpi	8.57	1.85	7.88	2.07	1.00	0.14	0.81	0.32	7.06	2.27	6.45	2.23	0.11	0.09	0.17	0.09
BALB/c 14 dpi	11.14	0.57	9.25	3.91	2.50	1.36	2.11	2.15	7.36	1.21	6.64	0.60	0.23	0.05	0.19	0.06
BALB/c 28 dpi	11.46	2.26	12.36	1.40	1.43	0.08	1.75	0.43	9.45	1.67	9.53	0.72	0.22	0.03	0.24	0.05
BALB/c 56 dpi	9.12	1.14	9.80	0.64	1.14	0.69	1.67	0.14	7.38	0.48	7.57	1.41	0.18	0.08	0.31	0.10
BALB/c 112 dpi	9.25	1.93	9.59	0.80	1.22	0.42	1.50[b]	0.21	8.02	1.38	7.15	1.18	0.11	0.04	0.12	0.02

The dpi at which the cell population was significantly ([a]$P < 0.05$; [b]$P \leq 0.01$) different between infested and uninfested (control) animals is shown. In addition, select values were outside of the published reference range. Monocyte populations were also evaluated; there were no significant differences between control and infested animals of any strain at any time point.
[c]Above upper limit of reference range (Charles River Laboratories, Clinical Pathology Data for North American CFW Mouse Colonies)
[d]Below lower limit of reference range (Charles River Laboratories, Clinical Pathology Data for North American C57BL/6NCrl Mouse Colonies)

Table 4. RBC, Hgb concentration, Hct, and reticulocyte count (median and IQR) for infested and uninfested (control) SW, B6, and BALB/c mice at 12, 28, 56, and 112 d after *Demodex* infestation (dpi; n = 5 mice/group/time point)

	RBC (× 10^3/µL)				Hgb (g/dL)				Hct (%)				Reticulocytes (× 10^3/µL)			
	Control	IQR	Infested	IQR	Control	IQR	Infested	IQR	Control	IQR	Infested	IQR	Control	IQR	Infested	IQR
SW 14 dpi	12.0	1.8	10.5	0.3	18.3	2.2	16.1	0.4	59.6	8.4	52.2	1.5	651.7	90.9	607.1	112.1
SW 28 dpi	11.10	0.7	10.4	0.5	16.8	0.3	15.2[a]	0.8	54.2	1.0	48.4[a]	1.7	653.8	59.3	501.8	190.7
SW 56 dpi	10.5	0.3	9.7	1.3	15.7	0.8	14.6	1.0	49.2	3.0	42.6	3.7	602.8	405.5	427.0	85.9
SW 112 dpi	10.9	1.0	10.5	0.4	15.8	0.7	15.6	1.8	48.8	2.9	49.3	4.5	587.5	155.3	622.1	182.3
B6 14 dpi	9.9	0.5	10.4	0.6	14.8	0.6	15.5	0.9	46.8	4.7	49.0	3.3	432.6	64.4	472.4	47.4
B6 28 dpi	10.1	0.3	10.0	0.2	14.4	0.3	14.4	0.1	45.3	0.5	45.9	0.5	351.2	89.4	344.7	41.7
B6 56 dpi	10.8	0.8	10.2[a]	0.3	15.1	1.2	14.8	0.2	48.2	3.7	45.5	1.5	1102.0	148.3	1183.0	123.9
B6 112 dpi	10.9	0.6	10.3	0.2	15.5	0.6	14.7	0.3	49.8	2.7	47.9	1.5	533.5	114.3	466.3	47.5
BALB/c 14 dpi	10.6	0.1	10.2	0.6	15.9	0.2	15.7	0.8	48.9	0.6	47.2	2.8	538.7	37.4	402.6[a]	32.1
BALB/c 28 dpi	10.9	0.7	10.7	0.2	16.6	0.8	16.4	0.5	48.4	2.9	47.9	2.2	544.7	116.7	574.8	47.3
BALB/c 56 dpi	11.0	0.5	11.1	0.7	16.8	0.8	16.3	0.8	49.3	3.6	49.0	3.0	489.6	104.9	486.4	150.8
BALB/c 112 dpi	13.0	0.2	11.6[a]	0.3	19.6	0.6	17.1[a]	0.8	60.2	2.9	52.6[a]	1.5	670.3	181.9	507.6[a]	61.6

Data are shown as dpi at which the cell populations were significantly ([a]$P \leq 0.05$) different between infested and uninfested (control) animals. All values were within published reference ranges.

Immunophenotyping. No consistent changes in immunophenotype were observed in infested mice of any strain between time points. Overall, a few significant differences were detected in cell marker expression; therefore we examined the distribution of cell types between infested and uninfested mice of the same strain at individual time points after infestation (Table 5). When statistically significant differences were detected, they were inconsistent—that is, the changes did not occur at all time points for a given strain. For example, the $CD8^+$ cell population was significantly smaller in infested BALB/c mice at 14 and 28 dpi (P = 0.012 and 0.037, respectively). The difference was not statistically significant at 56 dpi, and infested BALB/c mice had higher $CD8^+$ cell counts at 112 dpi. Similarly, few statistically significant differences in staining indices were detected for the T-cell activation markers CD25 and CD69 on $CD4^+$ and $CD8^+$ cells, and these changes were similarly inconsistent at the various time points analyzed (Table 6).

Serum IgE concentration. Serum IgE concentrations were higher in infested as compared with uninfested mice of the same strain at all time points (Figure 4 A). Infested BALB/c mice had the highest serum IgE concentration among the immunocompetent mice at all time points. Median serum IgE levels were significantly increased in BALB/c (28 dpi, P = 0.01; 56 dpi, P = 0.01; 112 dpi, P = 0.01) and B6 (28 dpi, P = 0.03; 56 dpi, P = 0.01; 112 dpi, P = 0.02) mice as compared with the uninfested (control) mice at 28, 56, and 112 dpi. Although IgE concentrations in BALB/c mice fluctuated considerably over time, the peak concentration occurred at 56 dpi, and the differences in median IgE levels between control and infested B6 mice were consistent at each of the last 3 time points.

Treatment. All infested, treated BALB/c mice (TG 1) were mite-free on histology and according to PCR analysis by 7 dpt. Of the infested, vehicle-treated BALB/c mice, 2 of 5 animals tested positive for *D. musculi* at 7 dpt. Of these 2 mice, 1 was

Table 5. The percentage (median) of parent population of B cells, CD4+, CD8+, neutrophils, and monocytes for SW, B6, and BALB/c mice at 14, 28, 56, and 112 d after *Demodex* infestation (n = 5/group/strain/dpi) as determined by flow cytometry

	B cells		$CD4^+$ cells		$CD8^+$ cells		$CD4^+$:$CD8^+$		Monocytes		Neutrophils	
	Control	Infested	Control	Infested	Control	Infested	Control	Infested	Control	Infested	Control	Infested
SW 14 dpi	65.60	60.70	22.30	23.30	3.74	4.47	5.22	4.27	42.90	42.10	17.10	22.50[a]
SW 28 dpi	77.40	75.30	14.10	15.30	3.03	3.37	4.65	4.51	20.50	19.10	13.80	15.70
SW 56 dpi	75.00	70.90	15.20	20.90	4.40	5.03	3.42	4.18	19.95	21.25	47.60	34.10
SW 112 dpi	73.10	76.40	15.30	15.50	3.39	3.23	3.53	4.80	32.30	31.60	45.60	35.80
B6 14 dpi	69.40	63.00	12.52	16.90	11.00	12.70	1.19	1.33	29.50	32.90	9.04	6.24
B6 28 dpi	60.00	44.10	14.20	22.20	18.60	22.20	0.85	1.08	26.80	36.70[a]	44.20	31.50
B6 56 dpi	54.60	51.40	16.30	18.60	18.20	14.40	0.90	1.27[a]	35.50	40.20	34.50	27.60
B6 112 dpi	60.50	60.00	13.90	14.00	12.50	13.20	1.11	0.96	25.70	24.90	24.40	28.20
BALB/c 14 dpi	48.80	62.30	19.10	17.50	20.30	12.80[b]	0.94	1.45	48.60	42.90	3.12	14.50
BALB/c 28 dpi	56.40	61.30	14.30	15.00	19.80	11.20[a]	0.91	1.59[a]	32.40	42.10	43.60	27.20
BALB/c 56 dpi	65.00	64.50	16.20	17.30	10.70	9.36[a]	1.52	1.83[b]	25.30	38.10	35.20	17.80
BALB/c 112 dpi	51.40	52.40	21.90	21.10	10.10	12.90	2.17	2.00	34.10	29.00	25.70	44.30

Data are shown as dpi at which the percentage of the cell population was significantly ([a]$P \leq 0.05$; [b]$P \leq 0.01$) different between infested and uninfested (control) animals. The percentage of $Cd11b^+$ cells were not significantly different between infested and control animals in any strain at any time point.

Table 6. T-cell activation as reflected by the Stain Index for $CD8^+CD25^+$, $CD8^+CD69^+$, $CD4^+CD25^+$, and $CD4^+CD69^+$ cells for SW, B6, and BALB/c mice at 14, 28, 56, and 112 d after *Demodex* infestation (median of n = 5/ group/strain/dpi) as determined by flow cytometry

	$CD8^+CD25^+$		$CD8^+CD69^+$		$CD4^+CD25^+$		$CD4^+CD69^+$	
	Control	Infested	Control	Infested	Control	Infested	Control	Infested
SW 14 dpi	23,467	19,320	8016	8668	53,606	32,573	19,048	19,403
SW 28 dpi	8475	9649	19,435	14,603	42,242	62,602	18,240	17,344
SW 56 dpi	11,244	9400	13,009	13,362	23,198	20,110	21,549	20,515
SW 112 dpi	15,761	35,670	15,635	14,220	16,220	19,713	9836	10,412
B6 14 dpi	12,127	8236[a]	15,161	10,915[a]	51,975	62,707	19,105	18,585
B6 28 dpi	5669	3649	16,703	12,443	47,200	38,114	22,112	18,886[a]
B6 56 dpi	28,054	29,573	9500	7696[a]	36,489	37,100	18,467	15,443[a]
B6 112 dpi	6954	7449	8873	9198	31,689	29,700	7925	8720
BALB/c 14 dpi	17,077	16,901	15,053	16,801	71,353	43,319	18,521	17,483
BALB/c 28 dpi	20,178	23,920	17,071	15,930	63,907	80,381[b]	23,196	23,065
BALB/c 56 dpi	6848	8900	10,989	9180[a]	53,779	45,742	11,559	10,365
BALB/c 112 dpi	19,274	31,238	9436	10,328	37,625	35,933	7561	7260

There were several time points where expression levels of activation markers were significantly different between infested and uninfested animals. ([a]$P \leq 0.05$; [b]$P \leq 0.01$)

PCR-positive, and mites were observed histologically in the other animal. Only a single infested, untreated mouse was PCR-positive at 28 dpt. Mites were not evident on histopathologic sections for either of the PCR-positive mice at 7 and 28 dpt.

IgE concentrations for all treatment groups at 7 and 29 dpt are shown in Figure 4 B. At 7 dpt, IgE concentrations of TG 1 (infested, treated) and untreated, infested animals were similar to pretreatment levels and each other, and IgE concentrations of TG 2 (infested, treated) were significantly lower than those of TG 1 (P = 0.01) and infested, untreated (P = 0.01) mice. By 28 dpt, the IgE concentrations (median [minimum, maximum], 141 [84, 214]) of TG 1 animals had decreased considerably from their 7 dpt levels (median [minimum, maximum], 247 [116, 303]); however, this difference was not statistically significant. At 28 dpt, IgE concentrations for TG 1 animals were also decreased when compared with infested, untreated animals (median [minimum, maximum], 207 [190, 239]); however, this difference also was not statistically significant. At this time, IgE concentrations of TG 2 animals (median [minimum, maximum]: 106 [52, 128]) had increased from their 7-dpt levels (median [minimum, maximum], 65 [45, 90]; P = 0.25) but remained significantly lower than measured in infested, untreated mice (P = 0.01).

Discussion

D. musculi is more prevalent in laboratory mice than previously recognized. Of animal or environmental samples submitted to 2 large diagnostic laboratories over several years, 4.38% to 4.76% were positive for *D. musculi* (data not shown). Whether it is a 'reemerging' or an 'emerging' agent can be debated as the development of caesarian-rederived mouse strains during the last half of the 20th century resulted in the global availability and use of *Demodex*-free strains and stocks in laboratory research for many years. We speculate that the limitations in *Demodex* diagnostics—which until recently required direct observation of the mite in skin sections, skin scrapes, fur plucks, or tape samples—in concert with the global trade in

genetically engineered mice over the past several decades are the major contributing factors to its (re)emergence. Failure to test and exclude infested animals in quarantine likely led to the introduction of *D. musculi* into colonies, and subsequently, the mite was likely spread within colonies through breeding. Testing for excluded agents in quarantine, rather than introducing the strain or stock after caesarian or embryo transfer rederivation, likely introduced *D. musculi* into colonies and was subsequently spread through breeding. As unique strains of mice were generated, crossed with other strains, and shared among institutions, the prevalence increased—and likely continues to increase. What remained unknown is whether an infestation could cause clinically silent changes, such as immunologic perturbations, that might influence experimental results precluding the animal's use for research. Therefore, we sought to determine the effects of *D. musculi* infestation on the immunophenotype of several commonly used strains and an outbred stock to ascertain whether infestation could confound research results. In addition, we sought to enhance our understanding of the biology of the host–mite relationship in the context of the host's predilection to mount either a Th1- or Th2-skewed, heterogeneous, or absent immune response, and we further examined the use of PCR testing to detect and quantify mite burden.

Uninfested and infested mice in the immunocompetent strains and stocks (B6, BALB/c, and SW) showed only limited differences in the leukograms and hemograms. When significant differences resulted, they were seldom outside of the published reference ranges. As examples, infested SW mice developed lymphocytosis (median, $11.6 \times 10^3/\mu L$; reference range, 2.98 to $8.57 \times 10^3/\mu L$) and B6 mice were leukopenic (median, $4.4 \times 10^3/\mu L$; reference range, 4.9 to $8.9 \times 10^3/\mu L$) at 28 dpi; however, these abnormalities were not evident at other time points. Demodicosis has been associated with eosinophilia in dogs,[3] yet this condition was not a consistent finding in the present study. Although eosinophil counts were often slightly higher in infested as compared with uninfested mice, this difference was significant only in SW mice at 28 dpi, and although the direction of the differences was consistent for SW mice at all time points, the direction of the differences was inconsistent over time among other strains. Eosinophil counts always fell within the published reference range for infested animals regardless of strain and time point, and eosinophils were not observed in association with mite-infested follicles in skin sections.

Similar to leukogram and hemogram data, few significant, albeit inconsistent, variations in cell population distribution or activation marker activity were detected in infested compared with uninfested mice as determined by flow cytometry. We aimed to broadly survey the animals' immunophenotype by using cell markers that have been altered in canine demodicosis.[48,49] $CD8^+$ T cells were significantly lower in infested as compared with uninfested BALB/c mice at the first 3 time points evaluated. Humans with demodicosis have evidence of immunosuppression, and there is evidence of localized immune suppression in infested dogs.[1,28,48,50] Our findings could be indicative of mite-induced immunosuppression, yet this alteration was not observed in any other strain or cell type, and mite burdens decreased temporally and were lowest in BALB/c mice after 28 dpi. Because our survey of the immunophenotype of infested mice was broad, it is possible that we missed subtle changes. Investigating cytokine expression or other markers may be necessary to elucidate the immunopathogenesis of murine demodicosis. Some of the statistically significant differences may reflect false positives identified by chance. Because of the limited sample size, we did not perform corrections for multiple hypothesis testing. We performed hypothesis testing with a false discovery rate of 5%. In other words, in the absence of any true differences in the underlying population, it is expected that 5% of all comparisons would be statistically significant due to chance. However, we may have missed immunologic changes due to the time points chosen, or there may be changes in the localized dermal immune response that would go undetected by using the methods in this study.

Although we found no substantial temporal or strain-related hematologic or immunophenotypic alterations, all infested immunocompetent mice had higher IgE levels as compared with uninfested controls. BALB/c mice developed the most profound humoral response to *D. musculi* with IgE concentrations as high as 15-fold greater in infested than uninfested BALB/c mice at 56 dpi. Similar trends were observed in both B6 and SW mice, although the magnitude of the increases were considerably less. The IgE response was not immediate, given that the first statistically significant differences were observed in the inbred strains at 28 dpi. Th2 cytokines such as IL4 and IL3[45] are known to switch antibody isotype from IgM to IgE[20] and induce allergic or atopic responses. Because BALB/c mice are known to have a Th2-biased immune response, it is not surprising that they mounted the most profound serum IgE response of the strains and stocks evaluated. A similar response to *Myocoptes musculinus* was reported in BALB/c mice, but severe skin pathology and hematologic abnormalities were observed.[23] Previous work established a threshold level of 81 ng/mL serum IgE for suspicion of a fur mite infestation in SW mice.[44] In the present study, all infested animals surpassed this threshold, whereas control SW and B6 animals did not. Uninfested BALB/c mice had baseline IgE levels that exceeded this threshold, indicating that their IgE threshold may be higher than that of other strains. Whether marked elevations in IgE alone are responsible for reducing mite burdens in BALB/c mice and, to some extent, the other strains and stocks remains unknown.

Clinical dermatologic changes were noted in the infested immunocompetent mice, even though their mite burdens were low. Alopecia was evident in the SW stock. In dogs with *D. canis*, there is evidence of apoptosis of hair follicle cells and follicular shrinkage,[53] and it is possible that a similar mechanism of alopecia occurs in some *D. musculi*-infested strains. B6 and BALB/c strains showed increases in lesions consistent with barbering, which is commonly reported in uninfested B6 mice.[4] Barbering has been associated with chronic mild stress several mouse strains, particularly BALB/c mice,[33] and has been shown to confound neurobehavioral studies.[24] Although NSG mice had a considerable mite burden that increased temporally, reaching almost 55 mites/cm at 112 dpi, the clinically observable dermatologic changes were limited to scruffy fur on the face and dorsal cervical region, leading to speculation that the changes observed in the immunocompetent strains resulted, at least in part, from the immune response and were not caused directly by the mites.

We evaluated whether mite burden varied among 4 strains and stocks. Burdens did differ, as expected, with the highest burden found in the highly immunocompromised NSG and the lowest in the Th2-skewed BALB/c strain, with Th1-skewed B6 mice having a slightly higher burden than the heterogeneous-responding SW stock. In dogs, systemic disease predisposes to *D. canis* infestation and increases in mite burden.[32] Experimentally naive healthy mice were used in the present study, so it remains unclear whether intercurrent disease or experimental stress would result in higher mite burdens.

Mite burdens in the immunocompetent mice were extremely low, which lead to false negative diagnostic test results,

especially when using methods that rely on direct mite observation. We found that skin histopathology was slightly more sensitive than PCR analysis. However, the histopathology method used involved examining multiple sections per mouse. Of the 480 total skin sections evaluated, approximately 60% did not contain mites, and examining 8 sections per animal is labor-intensive. Another group has previously shown that identification of mites in skin sections to be slightly more sensitive than PCR in detecting *D. musculi*, but that study evaluated an even greater number of sections per mouse.[39] The present study used a PCR assay with different proprietary primers. Of 122 assays performed, 2 samples yielded false-negative results: one from an SW mouse at 14 dpi, and another from an untreated BALB/c mouse at 7 dpt. Two untreated mice had positive PCR results, with no evidence of mites histologically. These results were perhaps not actual false positives, because PCR testing could have detected dead mites or parts of mites as they were being expelled from the hair follicle or were present on hair shafts, consistent with what has been reported previously for murine fur mites.[55] Therefore, differentiating active and resolving infestations by using PCR is difficult. The hair follicle cycle is approximately 6 wk in most mouse strains,[36] yet as long as 8 mo may be necessary for the entire pelage to be shed.[42] Therefore, mice may test PCR-positive for months after an infestation has resolved. Although testing of IVC filters and caging system components by PCR assay has proven effective for identifying the presence of fur mites,[17,21] these methods may not be as effective for *D. musculi* because it is poorly transmitted via dirty bedding.[37]

Mite burden did not correlate with PCR copy number in immunocompetent mice and thus cannot be used as a surrogate to determine the severity of infestation. Because PCR testing also detects mite fragments and debris, it is not surprising that copy number did not correlate with burden. However, we found a strong correlation between mite burden and copy number in NSG mice, perhaps because this strain harbored many more mites.

Histopathology findings suggest that *D. musculi* induces subtle dermatopathologic changes in the skin of BALB/c mice, principally acanthosis and hyperkeratosis, which are also frequently seen in *D. canis*-infested dogs.[3] Despite the absence of pruritus or crusting on gross examination, mites induced some damage to the epidermis and hair follicle, demonstrated as a higher frequency of alopecia in infested animals. Treatment appears to ameliorate these lesions. However, they seem to resolve spontaneously, albeit more slowly, in immunocompetent BALB/c mice when mite exposure ceases, given that there is a decline in mite burden resulting from the immune response. Notably, skin lesions resolve more rapidly than the serum IgE concentrations decreased. However, these findings are based on the BALB/c strain only, which mounts a robust immune response and may not be representative of other strains and stocks.

In dogs infested with *D. canis*, there is evidence of perifollicular pyogranulomatous inflammation in the dermis, likely because the mites are recognized as foreign bodies and to fend off secondary bacterial infections.[3] The major immunogenic component of *Demodex* spp. is unknown. In most mite allergies, the sensitivity is to the mite's excrement or an enzyme,[20] neither of which have been identified for *Demodex* species, although keratinocyte Toll-like receptor 2 recognizes mite chitin.[27,28,48] Although perifollicular inflammation is not observed in *D. musculi*,[37] we hypothesize that enzymes secreted by the mite during feeding may induce an immune response, as it does with other follicular parasites.[54]

Consistent with previous reports concerning the immunocompromised TRP strain,[37,38] imidacloprid–moxidectin was effective in immunocompetent BALB/c mice using the same dose and regimen. Immunocompetent mice may be capable of clearing the infestation without treatment. In our current study, 60% of untreated animals had no evidence of mites by 7 dpt, and this number increased to 80% by 28 dpt. Burdens may fall more rapidly depending on study design, for example, mite numbers would likely be higher in immunocompetent mice that were continuously housed with an immunocompromised strain, as we did for our mite burden and immunophenotyping studies. In contrast, the donor NSG mouse was removed in our treatment study. Treatment led to a marked decline in serum IgE, which, if we had continued to measure it, would likely have returned to baseline. This finding is similar to BALB/c mice treated with ivermectin for fur mites.[35] Although not statistically significant, IgE concentrations tended to decline in untreated BALB/c mice as their immune response led to a gradual reduction in mite burden over time. At least in those mice in which the immune response effectively cleared the mites, their IgE concentration likely could eventually return to baseline.

In conclusion, the principal aim of this study was to examine immunologic changes associated with demodicosis in order to determine whether using immunocompetent mice colonized with mites is a significant issue for the investigators using them. The answer remains unclear. Although immune responses and dermatologic changes occurred in immunocompetent strains, the magnitude of the immune response was insufficient to dramatically alter the mouse's immunophenotype. Until additional studies are conducted, a prudent approach is to avoid using *Demodex*-infested mice in studies affected by changes in skin immunology, particularly atopic dermatitis, or experiments evaluating the epidermal immune system.

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