Ivermectin-compounded Feed Compared with Topical Moxidectin–Imidacloprid for Eradication of *Demodex musculi* in Laboratory Mice

Melissa A Nashat,¹ Rodolfo J Ricart Arbona,^{1,2} Michelle L Lepherd,^{2,†} Sara F Santagostino,^{2‡} Robert S Livingston,³ Elyn R Riedel,⁴ and Neil S Lipman^{1,2,*}

Demodex musculi is a prostigmatid follicular mite that has rarely been reported in laboratory mice. Although prevalence of this species has not been assessed formally, we have found that many imported mouse strains from noncommercial sources harbor *Demodex* mites. To assess whether an acaricide can be used to eradicate this mite, infested immunocompromised mice were provided ivermectin-compounded (12 ppm) feed without restriction for 8 wk ($n = 10$), were treated topically with moxidectin and imidacloprid (MI; 3 and 13 mg/kg, respectively) weekly for 8 wk ($n = 10$), or remained untreated ($n = 10$). Mice were confirmed to be mite-infested before treatment and were tested after treatment by using fur plucks (FP), deep skin scrapes (DSS), and PCR analysis of fur swabs. In addition, the presence of mites was confirmed through skin biopsies at 2 study endpoints (1 wk [$n = 5$] and 12 wk [$n = 5$] after treatment). Samples collected before treatment and from untreated mice were positive for *D. musculi* at all time points and by all test modalities. After treatment, all ivermectin-treated animals remained infested, whereas mice treated with MI were repeatedly negative by all test modalities. An additional shortened treatment trial revealed that 4 wk of MI ($n = 7$) was insufficient to eradicate mites. Neither treatment produced any evidence of adverse effects according to hematology, serum chemistry parameters, behavior, body weight, and histopathology. Of the 70 PCR assays performed in treated mice, 14 were positive when FP+DSS was negative. In 6 cases where PCR results were negative, 5 were positive by FP+DSS and a single sample was positive on skin biopsy. Although PCR analysis has a high detection rate for *D. musculi*, FP+DSS can further enhance the detection rate. In conclusion, topical MI administered for 8 consecutive weeks can safely eradicate *D. musculi* from an immunocompromised mouse strain.

Abbreviations: DSS, deep skin scrape; FP, fur pluck; MI, moxidectin plus imidocloprid; NP, performed; NSG, NOD-*scid* gamma; NOD-*scid* IL2Rg^{null}, NOD-*scid* IL2Rgamma^{null}, TRP1/TCR: B6.Cg-Rag1^{tm1Mom}; Tyro1^{B-w} Tg(Tcra,Tcrb)9Rest/M (TRP1/TCR)

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Demodex mites (family Demodecidae, superfamily Cheyletoidea) are microscopic, cigar-shaped, prostigmatid mites that live, feed, and reproduce in the pilosebaceous units of mammals.^{11,22,33,69} They are present in healthy mammals, although these mites can become opportunistic parasites in immunocompromised hosts.^{18,22} *Demodex* mites are rarely reported in laboratory mice, but recent evidence suggests that they may go undetected and are likely more prevalent than previously recognized.^{32,55,67,90} Demodecid mites tend to be host-specific and, of the 6 species of *Demodex* mites identified in wild *Mus musculus*, only *Demodex musculi* has been reported in laboratory mice.^{14,32,37–40,55,67,90} Although the life cycle of *D. musculi* has not been characterized, the life cycle of human *Demodex* mites is estimated as 14.5 d according to in vitro experiments, and all life stages (eggs, larvae, nymphs, and adults) are found within hair follicles and sebaceous glands.^{69,92}

Although the effect of *Demodex* mites on the biology of immunocompetent mice has not been studied, treatment may be necessary in select immunodeficient mouse strains that develop clinical disease, or prior to introducing imported mice into established *Demodex* mite-free colonies.^{55,90} The effect of *D. musculi* on research has not been characterized in mice, but it is conceivable that these mites may perturb the immune and possibly other biologic systems.^{1,3,17,18,45,50} Vendor-sourced mice are unlikely to harbor ectoparasites, but many unique, genetically engineered strains are unavailable from commercial sources. Rederivation by embryo transfer or in vitro fertilization can be used to eliminate adventitious pathogens in mice, including ectoparasites, but it is costly and time-consuming.^{25,93,97} An effective method to eradicate *D. musculi* infestation would be beneficial for the maintenance of biosecurity in SPF colonies where mice are imported without rederivation.

Some acarine parasites in laboratory rodents can be eradicated by using antiparasitic treatments, but few studies are available regarding treatment options for *D. musculi*.^{55,90} Obstacles to treating follicular mites include achieving an effective concentration of the paraciticide at the skin's surface; the difficulty in confirming eradication after treatment due to the mite's small size and transparency; and, finding a treatment that has limited toxicity, is cost-effective, and is easily implementable on a large scale.^{12,17,55,74,79,90,100} Furthermore, eradication requires the

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¹Tri-Institutional Training Program in Laboratory Animal Medicine and Science and ²Center for Comparative Medicine and Pathology, Memorial Sloan Kettering Cancer Center, Weill Cornell Medicine, and The Rockefeller University, New York, New York;

³IDEXX BioResearch, Columbia, Missouri; and ⁴Epidemiology and Biostatistics Department, Memorial Sloan Kettering Cancer Center, New York, New York.

*Corresponding author. Email: lipmann@mskcc.org

[†]Current affiliation: Gribbles Veterinary, Christchurch, New Zealand

[‡]Current affiliation: Department of Safety Assessment, Genentech, Inc., South San Francisco, California

elimination of all *Demodex* mites, especially females, because parthenogenesis is a common reproductive strategy in mites, including demodicid species.^{52,69,70,98} In theory, a single female mite may repopulate its host. Thus, an effective treatment strategy must result in the eradication of all life stages.

A few studies have attempted *D. musculi* eradication by using the acaricide amitraz, but we opted to employ alternative strategies in the current trial.^{55,90} A topical combination of moxidectin and imidacloprid was recently demonstrated to be effective in dogs, and a commercial product (Bayer Advocate) is labeled for treatment of canine demodicosis in Europe.^{23,31,72,73} Imidacloprid, a potent neonicotinoid insecticide, acts as a nicotinic acetylcholine receptor agonist, interfering with neuronal transmission in invertebrates, and has a low propensity for toxicity in mammals.⁵⁹ Macrocytic lactones, including the avermectins (for example, ivermectin) and milbemycins (for example, moxidectin) are chemical derivatives from *Streptomyces* spp. These compounds bind to and thus activate glutamate-gated ion channels highly specific to nematodes and arthropods, resulting in hyperpolarization of neurons and flaccid paralysis, preventing parasite feeding and mobility.^{26,75} Because ivermectin is effective in treating canine demodicosis and because ivermectin has been used to eradicate rodent fur mites effectively, we decided to evaluate both oral ivermectin and topical moxidectin and imidacloprid (MI) for the treatment of *D. musculi* infestation in mice.^{15,35,48,79,100}

Recently, we characterized various parasitologic methods regarding the detection of *D. musculi* in laboratory mice.⁶⁶ In the current study, in addition to identifying an effective treatment regimen, we sought to further evaluate the *Demodex* mite detection rates of the diagnostic tests we used: fur pluck (FP) and deep skin scrape (DSS) examination, PCR analysis of fur swabs, and skin biopsy.⁶⁷ We defined *Demodex* eradication as negative results by all methodologies at the conclusion of the treatment trial. To our knowledge, this study is the first to critically assess ivermectin and MI as well as multiple diagnostic methods for the eradication of *Demodex* mites in mice.

Materials and Methods

Animals and husbandry. B6.Cg-Rag1^{tm1Mom} Tyrp1^{B-w} Tg(Tcr, Tcrb)9Rest/M (TRP1/TCR) female mice ($n = 40$; age, 4 to 7 wk) that were confirmed to be infested with *D. musculi*, were used for the treatment trials. We previously reported demodicosis in this strain.⁶⁷ In addition, for the 4-wk treatment trial, naïve *D. musculi*-free, female retired breeder NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice ($n = 11$; age, 4 to 7 mo) were used as contact sentinels to detect *D. musculi* infestation after treatment. Both strains were obtained from breeding colonies maintained at the Memorial Sloan Kettering Cancer Center (New York, New York).

TRP1/TCR mice were group housed ($n = 5$ per cage at the start of the experiment) in IVC (Thoren Caging Systems; Hazelton, PA) in a standard animal holding room. Cages were maintained under positive pressure with respect to the holding room. Feed was provided without restriction and included a closed-formula, γ -irradiated, natural-ingredient diet (PicoLab Mouse Diet 5053, Purina LabDiet, St Louis, MO) or an irradiated diet containing ivermectin (ivermectin-impregnated PicoLab Mouse Diet 5053 with 12 ppm ivermectin, TestDiet, St Louis, MO). Prior to treatment with parasiticides, all TRP1/TCR mice in both trials received enrofloxacin in the drinking water (85 mg/kg; 2.75 mL of 100 mg/mL enrofloxacin, Baytril, Bayer, Shawnee Mission, KS) in 473 mL of water for 2 wk, changed weekly, as prophylaxis for opportunistic infections, particularly *Pasteurella*

pneumotropica.^{27,67} Cages housing NSG mice after treatment (4-wk trial only) were provided amoxicillin-compounded diet (PicoLab Mouse Diet 5053 with 0.12% amoxicillin, TestDiet) to prevent opportunistic infections.⁶⁷ Once the treatment trial was initiated and thereafter, mice were provided acidified, reverse osmosis-purified water (pH 2.5 to 2.8) in 473-mL polysulfone bottles with neoprene stoppers (Techniplast, West Chester, PA), and they were housed in polysulfone cages (Thoren Caging Systems) with autoclaved aspen-chip bedding (PWI Industries, Quebec, Canada). For animals in the 8-wk trial, the entire cage (including cage bottom, bedding, wire-bar lid, and water bottle) was changed weekly within a vertical flow, HEPA-filtered, mass air displacement unit (model NU-S619-400, NuAire, Plymouth, MN). To prevent cross-contamination, different animal care technicians provided husbandry to colony and experimental animals, and cages were changed on different days.

After treatment with parasiticides, animals in the 4-wk trial were cohoused with NSG mice (1 TRP1/TCR and 1 NSG mouse per cage) in a separate holding room and the entire cage was changed weekly in a class II type 2A biosafety cabinet (model NU-S602-500, Nuair) by using strict microisolation technique to prevent cross-contamination between groups. During the 4 to 8 wk of treatment, cages were changed at the time of MI treatment. During the treatment trial, cages of MI-treated animals were handled first, then ivermectin-treated animals followed by untreated animals. Disposable gloves were changed between cages. The holding room was maintained on a 12:12-h light:dark cycle with room temperature of $72 \pm 2^\circ\text{F}$ ($22.2 \pm 0.5^\circ\text{C}$) and relative humidity ranging from 30% to 70%. IACUC approval for the maintenance and experimental manipulation of mice was obtained, and the institution's animal care and use program is AAALAC-accredited and operates in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th edition.³⁶

The biosecurity program at Memorial Sloan Kettering Cancer Center excludes the following pathogens: 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 musculinus* and *Radfordia affinis*), pinworms (*Syphacia* spp. and *Aspiculuris* spp.), and *Encephalitozoon cuniculi*. *Corynebacterium bovis* and *P. pneumotropica* are not excluded.^{16,67} Because TRP1/TCR and NSG mice are predisposed to infections with these opportunists, prophylactic antibiotics were administered as described.^{67,82}

8-wk parasiticide treatment trial (ivermectin and MI). Study mice were randomly assigned to the untreated, control group ($n = 10$), ivermectin feed treatment group ($n = 10$), or topical MI treatment group ($n = 10$). Mice were housed in distinct cages by treatment group to avoid direct contact and mite transmission between untreated and treated mice. The ivermectin-treated mice were provided with ivermectin-impregnated feed without restriction for 8 wk as previously described and subsequently were fed standard rodent diet beginning at week 9 after treatment.⁷⁹

The MI treatment group received a commercially available product containing MI dissolved in a proprietary hydrophobic vehicle that included an aromatic alcohol and a carbonate derivative (Advantage Multi, Bayer, Shawnee Mission, KS). The packaged vial of MI was diluted by a factor of 30 in 70%

ethanol and made fresh every 2 wk. Animals were weighed the day before treatment, and the dose was titrated to approximately 3 mg/kg moxidectin and 13 mg/kg imidacloprid. The calculated dose was split and administered topically between the scapulae and on the middorsum once weekly for 8 wk by using a single-channel micropipette (Pipetman Classic P100, Gilson, Middleton, WI). The volume of MI applied per mouse ranged from 59 to 74 μ L at week 1 to 63 to 86 μ L at week 8, depending on each animal's weight. Mice were monitored for behavior and adverse events as described later. The control group remained untreated for the duration of the experiment. Diagnostic samples (pelt swabs, deep skin scrapes, and fur plucks) were collected, as described later, midway through treatment (just prior to the fifth topical treatment) and at 1, 5, and 12 wk after treatment. Treated animals were handled prior to untreated animals to avoid cross-contamination of samples and subjects. Half of the mice in each group were euthanized 1 wk after treatment, and the remaining mice were euthanized 12 wk after treatment. At endpoints, mice were euthanized prior to sample collection. Euthanasia was performed by carbon dioxide asphyxiation in accordance with recommendations in the *AVMA Guidelines on Euthanasia of Animals*, 2013 edition.⁶ At euthanasia, the pelt was harvested and skin biopsies were collected from numerous sites for microscopic examination as described later.

4-wk parasiticide treatment trial (MI only). The trial was performed by using untreated controls ($n = 3$) and mice receiving 4 weekly topical MI treatments ($n = 7$) under the same treatment application protocol as described in the 8-wk trial (dose volume in the experiment ranged from 63 to 82 μ L). At the beginning of week 5 (1 wk after the last treatment), TRP1/TCR mice were separated from their original cage mates to prevent reinfestation if viable mites remained and were paired with a mite-naïve NSG contact sentinel (1 NSG and 1 TRP/TCR per cage). NSG mice were added as contact sentinels in the 4-wk trial because preliminary results from the 8-wk trial indicated they might be useful as an added means of mite detection, in that they would amplify mites in cases with low mite burden. NSG mice were placed into treated mouse cages prior to being placed in untreated mouse cages, to avoid cross-contamination. Because NSG mice are highly susceptible to opportunistic infections, mice were maintained on 0.12% amoxicillin-containing feed in lieu of standard rodent diet after cohousing with TRP/TCR mice. Diagnostic samples (pelt swabs, FP, and DSS) were collected from all TRP1/TCR mice at 1 and 16 wk after the last treatment, as described later. Additional FP and DSS were collected at 7 and 11 wk after treatment. NSG mice were sampled at 7, 11, and 16 wk after cohousing with TRP/TCR mice. Treated animals and their NSG cage mates were handled prior to untreated animals, as was done in the 8-wk trial. Prior to sample collection, mice were euthanized through carbon dioxide asphyxiation at 16 wk after treatment. At euthanasia, the pelt was harvested, and skin biopsies were collected from numerous sites for microscopic examination as described later.

Collection and processing of FP and DSS samples. FP and DSS samples were collected from the interscapular and caudal ventrum regions in conscious mice as described.⁶⁶ FP were performed by grasping several clusters of fur by using 3.5- to 4-in. Halstead curved mosquito forceps and rapidly extracting the hairs by pulling perpendicular to the skin. The forceps tip was immersed and rotated in 70% alcohol and then clamped on to a paper towel to remove residual hair and debris before sampling subsequent sites or animals. Forceps were scrubbed and cleaned with detergent and allowed to dry between time

points. FP from the interscapular and caudal ventral regions were combined on a single slide.

DSS was performed by firmly squeezing a region of skin ($1 \times 1 \text{ cm}^2$ to $1.5 \times 1.5 \text{ cm}^2$) with one hand and using a no. 10 scalpel blade with the other, making contact with the skin at a 30° to 45° angle and scraping with pressure against the grain of the hairs 3 or 4 times over the same region. Skin and hair debris from FP and DSS samples were placed in mineral oil on a microscope slide and coverslipped. FP and DSS were performed on the right and left sides of the midline, respectively. For each mouse, the location off midline of the sampling site was recorded, and alternating sides were used for subsequent time points. Disposable exam gloves were changed between animals prior to sampling. Samples were collected and slides were assessed by using trichoscopy by the same observer (MN) throughout the study. NSG mice were tested in the same manner to confirm they were free of *D. musculi* prior to cohousing and at specified time points. DSS from the interscapular and caudal ventral regions were combined on a single slide.

All FP and DSS samples were analyzed by a single observer (MN) by using light microscopy as previously described.⁶⁶ At all time points, the presence or absence of mites was recorded for comparison with other tests. In addition, the mites were counted, and the combined number of mites obtained on 4 tests (FP-interscapular, FP-caudal ventrum, DSS-interscapular, and DSS-caudal ventrum) was totaled and analyzed by treatment group. Data are presented in dot plots, such that each dot represents an individual value, the central line represents the median, and the whiskers represent the 25th and 75th quartiles. Graphs were produced by using a commercial application (Prism version 6.07 for Microsoft Windows, GraphPad Software, La Jolla, CA).

Topographical analysis of skin biopsies. A topographic approach was used to assess the skin for mites, as described.⁶⁷ At necropsy, after bacterial cultures and a pelt swab for PCR analysis were taken, FP and DSS samples were collected, and the pelt was subsequently harvested from both 8- and 4-wk trial animals by incising the skin along midline from the perineum to the ventral cervical region and making annular incisions around the carpi and tarsi, and tail base. The caudal vertebrae were sharply incised, and the pelt was retracted caudally to cranially. The palpebrae, vibrissae, and pinnae were sharply incised to remove the skin of the head and muzzle. The skin was mounted on a piece of cardboard ($8 \times 10 \text{ cm}$) and was fixed in 10% neutral-buffered formalin for at least 48 h. The skin sections were trimmed to 0.5 to 1 cm in length and 2 to 3 mm in width, depending on the anatomic location. A total of 24 skin sections from 12 anatomic regions (including the nose and vibrissae, interocular skin, pinnae, head, interscapular skin, cranium ventrum, midventrum, caudal ventrum, middorsum, caudal dorsum, limbs, and perineum) from each mouse were processed by standard methods, embedded in paraffin, and stained (section thickness, 4 μ m) with hematoxylin and eosin, and assessed for mites. The tail was omitted from analysis because mites are rarely detected in skin from the tail.⁶⁷ Skin sections were examined under $200\times$ to $400\times$ magnification. Samples were coded as either positive (+) or negative (–) for mites. In addition, for animals in the 8-wk trial, all transverse, longitudinal, and oblique sections of mites were counted, and the length of the skin section was recorded in millimeters. The number of mites per length of skin (in millimeters) was determined by dividing the number of mites by the length of the skin section.⁶⁷ Data are presented in dot plots (generated by using GraphPad Prism) such that each dot represents an individual

value, the central line represents the median, and the whiskers represent the 25th and 75th quartiles.

A full topographic analysis with mite counts was not performed in the 4-wk trial subjects. A total of 24 skin sections from 12 anatomic regions were harvested from each pelt of both TRP1/TCR and NSG mice. The sections were examined to determine whether mites were present or absent, but further characterization of the numbers of mites in skin sections was not performed as conducted in the 8-wk trial.

PCR sample collection and assay. Immediately prior to FP and DSS sample collection, samples for PCR were collected by rubbing an individually packaged adhesive swab (Pigeon Corporation, Tokyo, Japan) vigorously along the entire pelt, including the interscapular region, dorsum, axillary region, ventrum, the external ear canals, muzzle, head, and perineum. Visible hairs and debris were adhered to swabs after use. Swab tips were broken into 1.5-mL microfuge tubes and stored at -80 °C prior to testing. Samples were numerically coded (with treatment group identifiers removed) and mailed to IDEXX for testing.

Samples were analyzed by using a validated proprietary PCR assay (IDEXX Laboratories, Columbia, MO). Total nucleic acids were extracted from the swabs using a commercial kit (One-For-All Vet Kit; Qiagen, Valencia, CA). The real-time PCR assay targeted the *D. musculi* 18S ribosomal RNA genomic sequence deposited in GenBank (accession no. JF834894.1) using a minor groove binder hydrolysis probe (Life Technologies, Carlsbad, CA). The assay has an analytical sensitivity of between 1 and 10 template copies per PCR reaction. A hydrolysis probe-based real-time PCR assay targeting a housekeeping gene (conserved prokaryotic 16S rRNA gene) was used to confirm recovery of DNA in the test sample, to confirm nucleic acid integrity, and to ensure the absence of PCR inhibitors. Analysis and amplification of the product were performed using standard primer and probe concentrations with a commercially available master mix (LC480 ProbesMaster, Roche Applied Sciences; Indianapolis, IN) on a commercially available real-time PCR platform (LightCycler 480, Roche Applied Sciences).

The estimated copy number of *D. musculi* DNA in each PCR test was calculated by plotting the real-time crossing-point values from the *D. musculi* PCR assay on a standard curve generated by testing log-fold dilutions of a positive control with a known copy number.

Comparison of *D. musculi* detection methods. FP and DSS test results were combined and compared with results from PCR analysis and skin biopsy for each time point in the 8-wk trial. In addition, methods were compared by considering only the individual mice tested at specific time points. To determine whether combining multiple test methods increases mite detection, FP+DSS and PCR results were collectively compared with the skin biopsy results.

Weight assessment and observations. Treated mice and untreated controls were weighed prior to and weekly during parasiticide treatment and every 2 wk after treatment. Weights were used to calculate the appropriate dose of MI and assess treatment efficacy.

Routine cageside observations of mice were performed daily by animal care staff. For animals in the 8-wk trial, observations were made cage-side and any behavioral or clinical abnormalities were noted by a veterinarian (MN). Animals in the untreated and ivermectin-treated groups were also observed for 5 to 10 min weekly. Because the MI dosing regimen was novel, MI-treated animals were also observed for 5 min daily during the first 2 wk of treatment during the 8-wk trial. In addition,

MI-treated animals were observed cageside for 30 min after each topical treatment. Activities, including grooming, exploratory behavior, burrowing, eating and drinking, and resting, were recorded every 5 min during the 30-min observation and abnormal behavior, when present, was recorded.

Blood collection and clinical pathology. To assess whether changes in hematologic or serum chemistry parameters were present during treatment, blood was collected from the retro-orbital sinus of half the mice (that is, 15 of 30) immediately preceding the first treatment in the 8-wk treatment trial. Mice were anesthetized with isoflurane (1% to 4%), and blood was collected from the retro-orbital venous sinus. Because the blood volume collected depended on body weight, volumes collected were sufficient for either hematology ($n = 8$) or serum chemistry ($n = 7$) analysis. Blood was collected by using a 75- μ L micro-hematocrit capillary tube inserted into the medial canthus and delivered into either a microtainer tube with EDTA for hematology or a microtainer tube with silica-based clot activator for clinical chemistry (BD Microtainers, Becton Dickinson, Franklin Lakes, NJ). After blood collection, gentle pressure was applied to the medial canthus with gauze for 30 to 60 s. In addition, 1 mL 0.9% sterile saline was administered subcutaneously, and an ophthalmic lubricant (Puralube Vet Ointment, Dechra Veterinary Products; Overland Park, KS) was applied to the eye. The animals were observed until they recovered from anesthesia, at the end of the collection day, and 24 h later. A maximum of 1 mL blood was collected through cardiocentesis from all 8-wk trial animals immediately after euthanasia at the experimental endpoint.

CBC were determined from at least 100 μ L EDTA-treated blood by using an automated analyzer (ProCyte Dx Hematology Analyzer, IDEXX Laboratories, Westbrook, ME). Parameters assessed included RBC count, Hgb, Hct, MCV, MCH, MCHC, RCDW, reticulocyte count and percentage, platelet count, mean platelet volume, WBC count, neutrophil count, lymphocyte count, monocyte count, eosinophil count, and basophil count. In addition, manually prepared blood smears were examined.

For serum chemistry analysis, blood was centrifuged at 5000 rpm ($4696 \times g$) for 10 min. Serum was assessed for hemolysis, lipemia, and icterus and processed in an automated analyzer (AU680 Chemistry Analyzer, Beckman Coulter; Indianapolis, IN). Analytes studied included BUN, creatinine, ALP, AST, total bilirubin, total protein, albumin, globulin, phosphorous, total calcium, glucose, cholesterol, triglycerides, creatine kinase, sodium, potassium, and chlorine. When blood volume was insufficient (that is, 7 of 30 samples), select analytes (triglycerides, creatine kinase, Na, K, and Cl) were omitted from analysis.

Anatomic pathology and histopathology. All mice were submitted for necropsy. Mice in the 8-wk treatment trial underwent complete necropsies with pelt assessment, whereas mice in the 4-wk treatment trial underwent gross necropsies, and the entire pelt was harvested, sectioned, and evaluated microscopically as described earlier. Tissues collected and evaluated in the 8-wk trial subjects included the heart, lungs, trachea, thymus, tracheal lymph nodes, spleen, mesenteric lymph nodes, bone marrow, pancreas, adrenals, thyroid, salivary glands, esophagus, stomach, intestines, liver, gallbladder, kidneys, urinary bladder, uterus, ovaries, cervix and vagina, stifle joint, sternum, femur, vertebral column, skin (head and ventrum), and spinal column. Coronal sections of the head yielded tissues from the eyes, nasal cavity, oral cavity, ear canals, and brain. Tissues were fixed in 10% neutral-buffered formalin for a minimum of 48 h. Bones were decalcified by using a commercially available solution containing formaldehyde, formic acid, and methanol

(Decalcifier I solution, Leica Biosystems, Richmond, IL) for 72 to 96 h and then were profusely washed with tap water for 20 min. Tissues were embedded in paraffin, and sections (thickness, 4 μ m) were stained with hematoxylin and eosin. Histopathologic assessment was performed by a board-certified veterinary pathologist. Each set of skin sections, collected as described, was viewed by 2 evaluators (MN and a veterinary pathologist) to determine whether the sample was positive or negative for mites.

Bacteriology. Prior to treatment with enrofloxacin, the pelts and the oral and vaginal cavities of each mouse in the 8-wk trial were cultured for *C. bovis* and *P. pneumotropica*, respectively, by using sterile cotton tip applicators (BactiSwab, Remel, Lenexa, KS). Samples were plated on trypticase soy agar II with 5% sheep blood, Columbia colistin–nalidixic acid agar with 5% sheep blood, and chocolate II agar plates (BBL, BD Diagnostic Systems, Sparks, MD). Plates were incubated aerobically at 37 °C in 5% CO₂ for up to 7 d. Colonies were isolated and subcultured on TSA plates and incubated for 24 to 48 h. Subsequently, bacterial isolates were identified by using colorimetric biochemical tests (API Coryne and API 20 NE, bioMérieux, Marcy L'Etoile, France).

Statistical analysis. In the 8-wk trial, the numbers of mites obtained with FP+DSS at various time points, the PCR copy number averages, and the number of mites counted on skin biopsies were compared among subjects in the 3 groups (untreated, ivermectin-treated, and MI-treated) by using the Kruskal–Wallis test. Pairwise comparisons were performed by using the Wilcoxon Rank Sum test. At the endpoint for animals in the 8-wk trial, a Spearman rank correlation coefficient was calculated for the total number of mites detected on FP+DSS and in skin biopsies. The Wilcoxon Rank Sum test was used to compare differences between the numbers of mites detected by FP+DSS and PCR copy number averages for individual mice and between the differences in numbers of mites detected in skin biopsies and PCR copy number averages at different time points. For the 4-wk trial, mite counts on FP+DSS, mite counts from skin biopsies, and PCR copy number averages were compared between groups by using the Wilcoxon Rank Sum test. The mite detection rates (%) determined by FP+DSS, by PCR analysis, and in skin biopsies were calculated as the number of mite-positive animals divided by the number of tests performed at each time point and multiplied by 100%. When multiple tests were performed at a single time point on the same animal, test-specific detection rates or a combined rate for FP+DSS with PCR analysis were compared with the detection rate of mites on skin biopsy by using the McNemar test.

Weights were compared between treatment groups at 1 and 12 wk after treatment for both the 4-wk (2 groups by the Wilcoxon Rank Sum test) and 8-wk trials (3 groups by the Kruskal–Wallis test). Hematology and serum chemistry values were compared among treatment groups for animals in the 8-wk trial at 2 time points (1 and 12 wk after treatment) by using the Kruskal–Wallis test. *P* values less than 0.05 were considered significant. All analyses were performed by using SAS version 9.4 (SAS Institute, Cary, NC).

Results

Results from FP and DSS tests. FP and DSS were performed at various time points in both trials. All 40 infested mice tested positive by FP+DSS prior to treatment. Mites were counted in combined FP+DSS samples. The number of mites or eggs detected in pretreatment samples ranged from 1 to 43. None of

the 11 NSG mice tested positive for mites when tested prior to use as contact sentinels.

In the 8-wk trial, the number of mites detected among the 3 groups differed ($P < 0.05$) at each time point (Figure 1 A). The number of mites detected by FP+DSS in untreated mice increased over time. The mite yield from the ivermectin-treated mice was significantly ($P < 0.05$) lower than the untreated mice at both 1 and 12 wk after treatment, but not at midtreatment. A single transparent (dead) mite was found midtreatment by FP+DSS in 1 of the 10 MI-treated mice, but no mites were detected in any subsequent test. The number of mites detected by FP+DSS in MI-treated mice differed from untreated and ivermectin-treated mice at all time points (Figure 1 A). Test results at 5 wk after treatment were similar (data not shown).

In the 4-wk trial, mites were present by FP+DSS in all untreated animals ($n = 3$) at all time points, but were not detected on any MI-treated mouse ($n = 7$) at any time point (1, 7, 11, and 16 wk) after treatment. Therefore, the number of mites detected on FP+DSS from MI-treated mice (zero) was significantly lower ($P < 0.05$) than those counted in samples from untreated mice at each of the 4 time points after treatment.

The NSG mice cohoused with untreated mice in the 4-wk trial had detectable mites at 7 wk after initiation of cohousing and had high numbers of mites at 12 wk, with as many as 175 mites or eggs detected by FP+DSS at this time point. This number of mites or eggs was more than 4 times greater than that obtained from any untreated TRP1/TCR mouse at any time point. In addition, *Demodex* nymphs were observed in FP+DSS samples collected from NSG mice but were not detected in TRP1/TCR mice. *Demodex* mites were not detected by FP+DSS in NSG mice cohoused with MI-treated animals at any time point.

Detection of mites by skin biopsy. To maximize mite detection in treated animals, 24 skin sections from 12 distinct anatomic regions were assessed from each mouse to determine whether mites were present in the skin of untreated, ivermectin-treated, and MI-treated mice. In the 8-wk trial, mites were detected in all untreated and ivermectin-treated mice. For untreated mice, the number of mites per millimeter was higher at 12 wk than 1 wk after treatment (Figure 1 B), but this difference was not statistically significant. Mites were found in all skin regions assessed in untreated animals, but not all of the multiple sections from a particular region were mite-positive. Not all sections from the interocular region, ears, head, muzzle, and perineum were mite-positive in all infested animals. All sections from the interscapular region, cranial ventrum, mid- and caudal ventrum, mid- and caudal dorsum, and the limbs were mite-positive in all untreated animals in both the 8- and 4-wk trials.

In the 8-wk trial, the number of mites per millimeter of skin was significantly ($P < 0.01$) different among the 3 treatment groups at 1 and 12 wk after treatment (Figure 1 B). The number of mites was lower ($P < 0.01$) in ivermectin-treated mice as compared with untreated mice 1 and 12 wk after treatment (Figure 1 B). A single MI-treated mouse had 1 mite in the right forelimb at 1 wk after treatment, but all 5 MI-treated mice examined at 12 wk were mite-free (Figure 1 B). Untreated mice had significantly ($P < 0.01$) more mites per millimeter of skin as compared with MI-treated mice at 1 and 12 wk after treatment. Significantly ($P < 0.01$) fewer mites were detected in skin from MI-treated mice when compared with skin from ivermectin-treated mice at both 1 and 12 wk after treatment (Figure 1 B).

Of the 72 skin sections from untreated mice ($n = 3$) analyzed in the 4-wk trial, 62 (83.8%) were mite-infested. In contrast, 6 of the 7 MI-treated mice were mite-negative. The remaining MI-treated mouse had 4 mites in 3 (12.5%) of the 24 skin sections

Figure 1. (A) The number of mites detected through combined FP+DSS at various time points after treatment with ivermectin-containing feed or 8 weekly topical applications of MI. (B) The number of mites detected in 24 skin biopsies from 12 anatomic regions per mouse at 1 and 12 wk after treatment. *, $P \leq 0.05$; †, $P \leq 0.01$; ‡, $P \leq 0.001$; §, $P \leq 0.0001$; a, compared with untreated mice; b, compared with ivermectin-treated mice

(head, left forelimb, and cranial thorax) evaluated at 16 wk after treatment. Despite this result, MI-treated sections overall had significantly ($P < 0.05$) fewer mites than sections from untreated mice. In the 4-wk treatment trial, all of the NSG mice cohoused with untreated TRP1/TCR mice had mites. In particular, 2 of the 3 NSG mice had severe infestations, with almost every follicle or sebaceous gland containing 1 or more mites. The third NSG mouse, which replaced an NSG mouse that was euthanized due to diarrhea of unknown etiology, had fewer mites than the others. None of the NSG mice cohoused with MI-treated mice had detectable *Demodex* mites in skin sections, including the NSG mouse cohoused with the *Demodex*-positive MI-treated mouse. In addition to the 24 skin sections initially analyzed from this mouse, 15 additional skin sections from random regions of the remaining pelage were harvested, evaluated, and determined to be *Demodex*-free.

Correlation between mite counts on FP+DSS and the number of mites per millimeter of skin. The total number of mites in mouse skin sections evaluated at 1 and 12 wk after treatment in the 8-wk trial was strongly correlated to the total number of mites detected through combined FP+DSS tests of the same animal (Spearman coefficient, 0.91; $P < 0.0001$).

Comparison of results from PCR assay and diagnostic tests. Diagnostic test results for all mice enrolled in the 8- and 4-week trials are provided in Tables 1 and 2. All pretreatment pelt swabs

($n = 40$) were PCR-positive, in agreement with results from FP+DSS. In mice treated with ivermectin, PCR and FP+DSS results were in agreement midtreatment, but at 1 wk after treatment, PCR results from 3 of 8 mice were negative, although both FP+DSS and skin biopsy were positive. Two ivermectin-treated mice, which were PCR-negative at 1 wk after treatment, were positive by all methods when evaluated 12 wk after treatment. Importantly, all 5 ivermectin-treated mice in the 8-wk trial were positive by all methods at 12 wk after treatment (Table 1). Data at 5 wk were similar to those at 12 wk after treatment, except for a single PCR-negative, ivermectin-treated mouse that was positive by FP+DSS (not shown). Among MI-treated mice at the 8-wk trial midtreatment time point, 7 were PCR-positive but FP+DSS-negative, whereas the remaining one was PCR-negative but FP+DSS-positive. By 1 wk after treatment and thereafter, all 5 remaining animals tested negative by all 3 methods at 3 consecutive time points (Table 1).

A comparison of detection rates for the 4-wk trial revealed that at the end of treatment, fur swabs from 5 MI treated mice were PCR-positive, FP+DSS-negative similar to the midtreatment result in the previous trial (Table 2). At 7 and 11 wk posttreatment, all 3 control mice were positive on FP+DSS and all 7 MI-treated animals were negative. Sixteen weeks posttreatment, 2 MI-treated mice were positive by PCR, although both had tested PCR negative at the earlier time point. In contrast, all 5 mice testing PCR positive 1 wk post treatment were PCR negative 16 wk posttreatment. Interestingly, PCR and skin biopsies detected positive mice, which went undetected by FP+DSS in both the 8-wk (skin biopsy positive, FP+DSS negative; $n = 3$, 1 wk post-treatment) and 4-wk trials (PCR positive, FP+DSS negative; [$n = 5$] 1 wk and 16 wk [$n = 1$] posttreatment).

A comparison of *D. musculi* detection rates by method for the 8-wk trial is provided in Table 3. The PCR assay detected significantly more positive mice than FP+DSS at the midtreatment time point; the 3 test methods (FP+DSS, PCR and skin biopsies) did not differ at any other time point, although mites were detected in skin biopsies in more infested mice at 1 wk after treatment. When FP+DSS and PCR results were combined, the combination detected more infested mice than either test alone at 1 wk after treatment, but this result was not significantly different from the skin biopsy results (Table 3). There was full agreement among tests at the 12-wk time point.

Because mite burdens decreased after both ivermectin and MI treatment, we compared the various testing methods used. In both trials, 70 PCR assays tests were performed at multiple time points on treated animals (Tables 1 and 2). Of the 70 tests, 6 (8.6%; 4 in the ivermectin group and 2 in the MI treatment group) were negative by PCR assay, although mites were confirmed by another method. Of these 6 false-negative results, 5 were positive on FP+DSS, and the remaining 1 was positive on skin biopsy. Of the 70 PCR assays performed on treated animals, 14 were mite-positive by PCR but mite-negative according to FP+DSS. Skin biopsies were collected and evaluated in only 2 of these 14 cases (both were free of mites in biopsy samples); the other PCR swabs were collected prior to the animals' endpoints and biopsy collection. Whether they were infested with *Demodex* mites or the PCR amplified residual nucleic acid from decaying mites remains unclear. Of the 15 PCR tests performed on MI-treated animals that reached endpoints in both trials and for which there were corresponding skin biopsy results, only 2 (both in the 4-wk trial) were PCR-positive.

PCR product copy number in untreated compared with treated mice. Although the actual number of mites in a sample cannot be ascertained through PCR analysis, the number of

Table 1. Comparison of methodologies to detect *D. musculi* in untreated, ivermectin-treated, and moxidectin–imidacloprid-treated mice (8-wk trial)

Mouse no.	Pretreatment		Midtreatment		1 wk after treatment			12 wk after treatment		
	FP+DSS ^a	PCR ^b	FP+DSS	PCR	FP+DSS	PCR	Skin biopsy ^c	FP+DSS	PCR	Skin biopsy
Untreated										
1	+	+	+	NP	+	NP	+	PE	PE	PE
2	+	+	+	NP	+	+	NP	+	NP	+
3	+	+	+	NP	+	+	NP	+	+	+
4	+	+	+	NP	+	+	NP	+	+	+
5	+	+	+	NP	+	NP	+	PE	PE	PE
6	+	+	+	NP	+	+	NP	+	+	+
7	+	+	+	NP	+	+	+	PE	PE	PE
8	+	+	+	NP	+	+	+	PE	PE	PE
9	+	+	+	NP	+	+	+	PE	PE	PE
10	+	+	+	NP	+	+	NP	+	+	+
Ivermectin										
1	+	+	+	+	+	+	NP	+	+	+
2	+	+	+	+	+	+	NP	+	+	+
3	+	+	+	+	—	NP	+	PE	PE	PE
4	+	+	+	+	+	+	NP	+	+	+
5	+	+	+	+	—	NP	+	PE	PE	PE
6	+	+	+	+	+	—	+	PE	PE	PE
7	+	+	+	+	+	—	NP	+	+	+
8	+	+	+	+	+	—	NP	+	+	+
9	+	+	+	+	+	+	+	PE	PE	PE
10	+	+	+	+	+	+	+	PE	PE	PE
Moxidectin–imidacloprid										
1	+	+	—	+	—	—	NP	—	—	—
2	+	+	—	—	—	NP	—	PE	PE	PE
3	+	+	+	—	—	—	NP	—	—	—
4	+	+	—	+	—	—	NP	—	—	—
5	+	+	—	+	—	NP	—	PE	PE	PE
6	+	+	—	—	—	—	—	PE	PE	PE
7	+	+	—	+	—	—	+	PE	PE	PE
8	+	+	—	+	—	—	NP	—	—	—
9	+	+	—	+	—	—	—	PE	PE	PE
10	+	+	—	+	—	—	NP	—	—	—

NP, not performed; PE, previous endpoint.

^aThe presence of *Demodex musculi* mites or eggs indicates a positive FP+DSS result.^bFor PCR analysis, when the estimated copy number met or exceeded the test threshold, the test was marked as a positive result.^cFor skin biopsy, when mites or eggs were observed in any skin section ($n = 24$) from the subject, the result was recorded as positive.

gene copies present in a sample can be estimated. We hypothesized that when more mites are present in a sample, the PCR copy number will be proportionately higher, that is, samples from untreated mice would yield higher copy numbers than samples from treated mice. PCR product copy number generally increased between midtreatment and 12 wk after treatment in samples from untreated and ivermectin-treated mice (Table 4), but this increase was not statistically significant. PCR assays were not performed on untreated mice 5 wk posttreatment in the 8-wk trial, but ivermectin-treated mice ($n = 5$) had low copy numbers (range, 0 to 28), and all MI-treated mice were PCR-negative. In the 8-wk trial, copy numbers differed among untreated animals and the 2 treatment groups at both 1 and 12 wk after treatment (Table 4). In the 4-wk trial, the estimated copy number was not significantly different between untreated and MI-treated mice at either time point (Table 5). Notably, the interquartile range was variable in untreated and ivermectin-treated subjects.

Weight and behavioral assessment. All mice gained weight throughout the evaluation period, and body weight did not differ between treatment groups and untreated controls at 1 and 12 wk after treatment. Weights for mice in the 8-wk treatment trial are provided in Figure 2. Similarly, in the 4-wk trial, there were no differences in weight between the untreated and MI-treated animals 1 and 16 wk posttreatment (data not shown).

No abnormal behaviors were displayed by mice in any group. Animals treated with topical MI showed increased grooming of the dorsum for several minutes after its application, and mice that underwent skin scraping (in all groups) displayed burrowing behavior immediately after sample collection.

Hematology and serum chemistry analyses. Pretreatment hematologic parameters and serum chemistry analyte values were within normal ranges, as compared with published ranges for mouse strains (data not shown), except that a single mouse had an elevated AST.^{56,58,86} No hematologic parameter differed among the 3 groups at 1 or 12 wk after treatment (data not

Table 2. Comparison of diagnostic methods to detect *D. musculi* in untreated and moxidectin–imidacloprid-treated mice (4-wk trial)

	Pre-treatment		1 wk after treatment		16 wk after treatment		
	FP+DSS ^a	PCR ^b	FP+DSS	PCR	FP+DSS	PCR	Skin biopsy ^c
Untreated							
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
Moxidectin–imidacloprid							
1	+	+	—	+	—	—	—
2	+	+	—	—	—	+	+
3	+	+	—	+	—	—	—
4	+	+	—	+	—	—	—
5	+	+	—	—	—	+	—
6	+	+	—	+	—	—	—
7	+	+	—	+	—	—	—

^aThe presence of *Demodex musculi* mites or eggs indicates a positive FP+DSS result.

^bFor PCR, when the estimated copy number met or exceeded the test threshold, the result was marked as positive.

^cFor skin biopsy, when mites or eggs were observed in any skin section ($n = 24$) from a subject, the result was recorded as positive.

Table 3. Detection rates of various combinations of diagnostic methods at multiple time points (8-wk trial).

	Midtreatment ^a		1 wk after treatment ^a			12 wk after treatment ^a		
	FP+DSS	PCR	FP+DSS	PCR	Skin biopsy	FP+DSS	PCR	Skin biopsy
No. of positive tests / total no. of tests ^b	11/20	17/20	8/15	5/9	11/15	10/15	9/14	10/15
Detection rate (%)	55	85 ^c	53	56	73	67	64	67
No. of positive tests / total no. of tests	NP		FP+DSS+PCR		Skin biopsy	FP+DSS+PCR		Skin biopsy
Detection rate (%)	NP		6/9		7/9	9/14		9/14
	NP		67		78	64		64

NP, not performed

^aThe detection rates of FP+DSS or FP+DSS plus PCR were compared with the rate of skin biopsy at midtreatment, 1 wk after treatment, and 12 wk after treatment by using the McNemar test.

^bResults from individual mice (positive tests) were analyzed when the mice underwent all of the tests being compared.

^cSignificant ($P < 0.05$) difference between FP+DSS and PCR analysis at midtreatment.

Table 4. Estimated *D. musculi* PCR copy numbers obtained from pelt swabs at various time points in the 8-wk treatment trial

Treatment group	Pretest	Midtreatment			1 wk after treatment			12 wk after treatment		
	All	Untreated	Ivermectin	MI	Untreated	Ivermectin	MI	Untreated	Ivermectin	MI
N	30	10	10	10	8	8	8	4	5	5
Median estimated copy number	287	NP	26.0	1.5	855.5	8.0	0.0	2883.0	92.0	0.0
(IQR)	(157.5–482.5)		(7.8–88.5)	(0.0–5.0)	(258.3–1614.0) ^b	(0.0–43.0) ^b	(0.0–0.0) ^b	(932.0–5494.0) ^a	(32.0–449.5) ^a	(0.0–0.0) ^a

At the designated time point, values were significantly (^a $P \leq 0.01$, ^b $P \leq 0.0001$; Kruskal–Wallis test) between groups.

Table 5. Estimated *D. musculi* copy numbers obtained in PCR assays of pelt swabs at various time points during the 4-wk treatment trial

	Pretest	1 wk after treatment		16 wk after treatment	
	All	Untreated	MI	Untreated	MI
N	10	3	7	3	7
Median estimated copy number (IQR)	818.4 (422.4–3975.0)	260.0 (9.0–498.0)	1.0 (0.0–9.0)	8960.0 (17.0–9184.0)	0.0 (0.0–6.0)

Values were compared between groups using the Wilcoxon Rank Sum test.

Figure 2. Body weights of untreated and treated mice before, during (bar), and after treatment (8-wk trial). Data are given as mean $\pm$ SEM ($n = 5$ to 10 per group). Time 0 indicates the start of treatment. Weight did not differ significantly between groups.

shown). However, several statistically significant ($P < 0.05$) differences in select serum chemistry analytes emerged among the 3 groups (data not shown), with mice receiving MI having slightly elevated BUN at 1 wk after treatment (compared with both untreated and ivermectin-treated mice) and slightly increased ALP at 12 wk posttreatment (compared with ivermectin-treated but not untreated mice). In contrast, compared with all other groups, ivermectin-treated animals had mild increases ($P < 0.05$) in total protein, due to elevated albumin, and cholesterol at 1 wk after treatment. Ivermectin- and MI-treated animals had slightly higher ($P < 0.05$) triglyceride levels at 12 wk after treatment. All values observed, including those for which differences were statistically significant, were within the reported ranges for the specific analytes in mice.^{56,58,86}

Anatomic pathology. Orthokeratotic hyperkeratosis was observed in skin sections from several mice in all groups; it was mild in ivermectin- and MI-treated mice and moderate in untreated mice. All untreated mice had multifocal sebaceous gland hypertrophy, whereas hypertrophy was observed in only 3 of the 10 ivermectin-treated and in none of the 10 MI-treated mice. Of the 30 mice in the 8-wk trial, 29 had lymphoplasmacytic, eosinophilic, or neutrophilic dermatitis; however, the lesions in the MI-treated mice were subjectively less severe than in the untreated and ivermectin-treated animals. In addition, mice in the 4-wk trial, which were maintained on an amoxicillin-containing diet, had subjectively less dermatitis than animals in the 8-wk trial.

Select histopathologic lesions were observed in a variety of tissues from mice in the 8-wk trial, but the incidence did not differ among groups. Lesions were attributed to the species (for example, extramedullary hematopoiesis), genetic background (for example, lymphoid depletion), or age (for example, ovarian atrophy). The untreated animal that died prematurely in the 8-wk trial had metastatic thymic lymphoma.

Bacteriology. From the samples collected pretreatment ($n = 30$) and at study endpoint ($n = 29$, excluding the mouse that died prematurely due to thymic lymphoma), neither *C. bovis* nor *P. pneumotropica* was cultured.

Discussion

This study was undertaken to determine whether eradication of *Demodex* mites from infested immunocompromised mice was

possible. We sought a treatment regimen that was easily administered, had minimal biologic effects, and was inexpensive with regard to labor and materials. We desired a treatment regimen that could be used prophylactically to treat rodents imported from noncommercial sources, principally other academic colonies, which require quarantine, because we have observed that a considerable proportion (approximately 13%) of imported immunocompetent strains harbor *Demodex* mites.⁸⁰ Our work is timely, given that a recent survey of 63 institutions in the United States that reported outbreaks of adventitious agents indicated that 8% ($n = 5$) of the institutions incurred outbreaks with *Demodex* mites.⁵⁷ In the current report, we demonstrated that *D. musculi* can be eradicated from an immunocompromised mouse strain with a moderate mite burden by using a combination of moxidectin (a milbemycin endectocide) and imidacloprid (a neonicotinoid pesticide).

Although demodicosis is rarely reported in laboratory mice, it can occur in any immunocompromised mammal, with dogs being the best studied veterinary species. Systemic and topical treatment for demodicosis in dogs was first reported in 1965.⁶⁹ A recent meta-analysis citing 106 clinical trials comprising more than 3400 dogs and assessing more than a dozen treatment combinations and intervals concluded that topical moxidectin is a preferred treatment.⁷ In addition, although amitraz and ivermectin have been used historically and are sometimes effective, numerous cases were refractory to treatment, and toxicity has been observed with both compounds, especially in particular dog breeds.^{7,63} A recent retrospective study reported a relapse rate of 11% in pet dogs treated for demodicosis with various agents.¹³

In rodents, including laboratory mice, treatment for demodicosis with amitraz has been attempted.^{30,43,55,90} In one study, amitraz was allegedly effective; however, in a more recent study, it failed to eradicate *D. musculi*.^{55,90} Given amitraz's potential toxicity, risk of occupational exposure, and labor-intensiveness of treatment (involving bathing individual mice biweekly), we sought alternatives.^{4,5,20,99} Avermectins, such as selamectin and doramectin, have been recommended or used to treat fur mites and *Demodex* spp. in dogs and rodents; however, reports indicated a limited success in relieving clinical signs; therefore, we did not consider them.^{7,19,29,62-64,81} We chose ivermectin because it has been used successfully to treat canine demodicosis and fur mite infestations in laboratory mice.^{7,15,64,79,100} In addition, ivermectin (oral or injectable) has been recommended or used to treat demodicosis in rodents, including hamsters and gerbils, in companion animal practice; it is relatively inexpensive; and it has successfully been administered in feed to treat murine fur mites in a large colony.^{34,79,94} In addition, we selected MI for study because this combination is effective in treating both canine and feline demodicosis when administered weekly.^{23,73,87} Moxidectin alone has been used to treat a murine fur mite infestation, but combinations of MI have been evaluated only experimentally to treat murine nematodes, and, to our knowledge, weekly treatments for an extended duration have not been evaluated in mice.^{62,74,76,85}

Our primary aim was to identify a regimen that would eradicate *D. musculi* mites in mice, defined as no detectable mites by any method at the conclusion of the trial. The presence of even a single mite after treatment potentially could result in repopulation because of mite asexual reproductive strategies (parthenogenesis). In other mammalian species, the treatment goal is to reduce the clinical signs related to demodicosis. For example, hamsters were considered 'cured' when all mite-associated dermatologic lesions were resolved and DSS were

mite-negative for 3 mo.⁹⁴ In dogs, after a course of treatment for demodicosis, 2 consecutive mite-negative DSS, taken 1 mo apart, indicated a 'parasitologic cure,' and treatment for 1 mo beyond the second set of negative tests was recommended.^{64,71,72} Some dogs have a clinical response to therapy but never achieve parasitologic cure, and treatment is recommended for life.⁶⁴ In general, after a clinical response, canine patients should be monitored for 1 y beyond treatment to assess for relapse.⁶⁴

Our results show that ivermectin significantly reduced the *Demodex* mite population according to FP+DSS mite counts and skin biopsy, but a dose of 12 ppm ivermectin did not result in mite eradication. We did not consider a higher dose of ivermectin because of reported toxicity.^{77,89,95} According to the authors of one study, 10 mice coinfecting with fur and *Demodex* mites and that received ivermectin (1%; 7 µg/mL) administered in the drinking water for 5 d were cleared of all acarine parasites.²⁸ Whether *Demodex* mites were fully eradicated is unclear, because the mice were tested only once, by skin scrape at 10 d after treatment, but the exact methodology was not indicated.²⁸ Oral and subcutaneously administered ivermectin have been used to treat hamster demodicosis and idiopathic dermatitis. Although clinical improvement was observed in some cases, some animals required an extended treatment course, whereas others relapsed or did not show improvement.^{54,94} Importantly, several institutions, including ours, currently administer ivermectin-containing feed to mice in quarantine to eradicate fur mites, which can go undetected, but because this practice results in reduction of *Demodex* mites (as shown in the current study), it likely makes their detection even more difficult.

In the current study, 5 of the 10 mice treated with MI weekly for 8 wk were evaluated as late as 12 wk after treatment and were mite-negative at 3 consecutive time points, according to FP+DSS, PCR assay, and the evaluation of an extensive number of skin biopsies. However, in the 5 MI-treated mice evaluated earlier (at 1 wk after treatment), a single mite was observed in a single skin section from a single mouse. Although we cannot state with absolute certainty, we strongly suspect that this mite was nonviable and had not yet been shed. Our attempt to reduce the number of treatments to 4 wk was unsuccessful, because 2 of the 7 MI-treated mice remained PCR-positive, 1 of which was confirmed in skin biopsies. Whether a treatment regimen involving more than 4 but fewer than 8 topical weekly MI treatments would be effective remains unknown, but it is likely, given that only a few mites were found in a few sites in the single mite-positive mouse in the 4-wk trial. Conveniently, for eradication of other murine mites, such as fur mites, a single dose of moxidectin is sufficient.⁷⁶ Therefore, regarding the importation of *Demodex*-free mice, topical MI should be considered as a preferred treatment protocol over ivermectin, and this regimen can target other ectoparasites as well. Of note, the MI combination is recommended to treat mild to moderate infestations with *Demodex* in other species.⁷ It remains unknown whether 8 wk of MI would be sufficient to eradicate the mites in heavily infested mouse strains.

Although an 'inhouse PCR assay' showed good agreement with FP+DSS, commercial PCR assays for *Demodex* have not previously been evaluated or compared with traditional methods of mite detection.⁶⁶ Our data demonstrate that the detection rate for *Demodex* mites of FP+DSS was not as high as multiple skin biopsies in mice with a low mite burden; however, there was a positive correlation between mites observed on FP+DSS skin tests and skin biopsy. PCR analysis is a valuable diagnostic method because it is highly sensitive for the detection of *Demodex* mites, with only an individual mite required for

a positive reaction.^{66,83,101} Our results reflect that commercial PCR analysis has a high detection rate, because 14 of 70 assays in treated mice were PCR-positive whereas FP+DSS remained negative. Although FP+DSS is the preferred method for ante-mortem *D. musculi* detection in dogs and other species, negative results in any species should be interpreted with caution.⁶⁴ Our data indicate that FP+DSS can be useful in conjunction with PCR assay, because FP+DSS detected mites in 5 animals in which PCR assays were negative. Advantages of FP+DSS include cost-effectiveness and that results can be obtained quickly. For FP+DSS, correct sample collection is important. Disadvantages of the tests include that training is required for sample assessment, careful assessment of each sample is necessary, and samples should not be pooled. Although differences in combined test results of FP+DSS and PCR analysis did not reach statistical significance compared with skin biopsy, likely due to small sample size, trends were similar in both treatment trials. Therefore, we recommend the combination of FP+DSS and PCR assay to maximize *D. musculi* detection, because collectively these analyses detected a greater number of infested animals than either test alone.

As occurs with FP+DSS, the detection rate of PCR analysis was reduced as the mite burden decreased with treatment in ivermectin- and MI-treated mice. This effect is a concern because immunocompetent mice likewise have low mite burdens. In addition, mice that test PCR-positive but FP+DSS-negative after treatment might not have been true false-negatives, because mite nucleic acid can be present without viable mites or visible mite structures. We found 1 dead mite by FP+DSS in 1 mouse at midtreatment in the 8-wk trial. Therefore, nonviable mites and mite debris likely contribute to the nucleic acid pool, explaining why 5 of 7 mice were PCR-positive but FP+DSS-negative at 1 wk after treatment in the 4-wk trial. This finding indicates that 1 wk after treatment is too early to use PCR analysis to verify treatment success. Interestingly, we observed that PCR copy numbers declined rapidly, in many cases to 0, in treated mice, thus supporting a treatment response to both ivermectin and MI treatment.

The chance for relapse in treated companion animals and the PCR-positive results at 1 wk after treatment in our mice provide insight regarding when treated mice should be tested for mites. Multiple mite life cycles should transpire prior to initiating testing. The life cycle of *D. musculi* has not been fully characterized, but is likely similar to that of *D. folliculorum* (14.5 d in length), although longer life cycles have been postulated for some rodent *Demodex* mites.^{68,92} We recommend beginning testing 12 wk after treatment, because this interval is sufficient for dead mites to have been shed in terms of the hair follicle cycle and completion of approximately 6 mite life cycles.^{78,92} When assaying colonies to determine whether *Demodex* mites are present, we recommend direct testing of colony animals as well as soiled-bedding sentinels, given that our experience is that these sentinels are not sufficiently sensitive to detect *Demodex* infestation in a large population. Additional testing strategies to increase the likelihood of mite detection include testing multiple animals in a cohort or of a specific strain and pooling samples for PCR analysis and testing specific animals at multiple time points. Testing IVC system components has been used successfully to detect other pathogens, including fur mites.^{9,44} In our experience, it is possible to detect *Demodex* mite nucleic acid in IVC system exhaust filters and on filter tops from cages housing *Demodex* infected mice, and it remains plausible that testing of caging system components will be useful for *Demodex* mite surveillance in colony health monitoring programs.

We expected that using highly immunocompromised NSG mice as contact sentinels would amplify viable mites and enhance detection. We previously observed the transfer of *Demodex* mites from infested adult TRP1/TCR mice to NSG mice by direct contact; a 12-wk postexposure period was sufficient for mite overgrowth in NSG mice cohoused with untreated animals, as was done in the current study. None of the NSG mice cohoused with treated animals in the 4-wk trial were positive for *Demodex* mites. Unfortunately, the single NSG mouse cohoused with a confirmed positive MI-treated mouse was mite-negative on 39 skin biopsies. This finding may reflect that direct contact is less efficient when low mite burdens are present, that *Demodex* mites may only transfer from a host when a minimum population density threshold is reached, that the mites had a preference for TRP1/TCR mice, or that the mites detected in the MI-treated mice were not fecund or viable. We think it is unlikely that the detected mites were nonviable or sterile, because mites were present in skin sections collected from a mouse euthanized 16 wk after treatment and, if nonviable, these mites would have degraded considerably or would have been shed during this period. Therefore, NSG mice may not be useful as contact sentinels for *Demodex* mites in a quarantine setting, because we suspect that many immunocompetent mice have very low burdens of mites.

Assessment of skin biopsies involved collection and evaluation of numerous tissue sections from multiple anatomic regions. Routine histologic assessment of skin sections might reveal mites, but the review of a limited number of sections is not as sensitive as examining a large number of sections.²¹ Although the 24 skin sections we examined represent only a fraction of the entire mouse pelt, our prior study indicated that approximately 3500 to 4000 follicles are assessed, and this method was highly efficient for detecting mites in TRP1/TCR mice.⁶⁷ Disadvantages of analyzing multiple skin biopsies include that it is both labor- and time-intensive and should be performed postmortem. Although skin biopsies can be harvested from live mice, this technique is invasive, and only 1 or 2 samples should be collected antemortem, potentially decreasing the method's sensitivity. Whereas *Demodex* mites are found throughout the pelt, some areas, such as the interscapular region, are more prone to infestation and should be biopsied preferentially.⁶⁷ When evaluating skin sections from mice, it is important to note that sebaceous gland hypertrophy is potentially indicative of a *Demodex* infestation, even when mites are not visible in the section. Sebaceous gland hypertrophy was present in all samples from untreated mice and in some from ivermectin-treated mice but not in MI-treated skin. Pathologists should suspect mites when gland hypertrophy is present. In contrast, orthokeratotic hyperkeratosis, a nonspecific finding, was seen in only a limited number of mice in this study, compared with observations from mice in this colony previously.⁶⁷ This difference is likely because mice in the current study received antibiotic prophylaxis to prevent infections by opportunistic pathogens, including *C. bovis*, which can cause extensive hyperkeratosis.

Mite counts in skin biopsies trended higher in untreated mice at 12 wk as compared with 1 wk after treatment. Similarly, mite yields in FP+DSS and PCR copy numbers trended higher in samples collected at later time points. Although comparisons among different methods at various time points were not statistically significant, likely because of small group sizes, more mites were found at later time points, suggesting that mite burdens increase as immunocompromised mice age.⁶⁷ *Myocoptes musculus* burdens also increase over time in Rag1-null mice as compared with immunocompetent strains.⁶¹

The PCR copy numbers in untreated mice were lower when mice were younger and presumably had a low mite burden, but the trend was for copy number to rise over time. In ivermectin-treated mice, the PCR copy number fell during treatment, but afterward it rebounded. We expected the mite counts—and PCR copy numbers—from the skin of mice treated with ivermectin to be higher at 12 wk as compared with 1 wk after treatment, because there would have been sufficient time for the mite population to expand. Although fur swabs collected from selected mice had higher copy numbers (note the interquartile ranges in Table 4), the overall lower-than-expected mite counts observed histologically might reflect that female mites are more sensitive to ivermectin, that ivermectin might impair mite fecundity, or that the immune system—even in an immunocompromised strain—may be better able to tolerate and regulate smaller numbers of mites, because host immunity appears to inhibit mite proliferation without visible signs of inflammation.¹⁸ For mite populations to rebound to pretreatment levels, a duration longer than 12 wk may be required.

MI-treated mice yielded a low *Demodex* copy number at midtreatment, with no detectable DNA at 3 consecutive time points, thus indicating that PCR analysis is valuable in confirming treatment efficacy. As expected, untreated control mice had higher mean copy numbers than those in both treatment groups in the 8-wk trial, and although this pattern was observed in the 4-wk trial, it did not reach significance. The small sample size likely contributed to the lack of statistical significance. Although all mice were swabbed vigorously, yielding swabs with visible debris, and although all control mice were PCR-positive, some samples might have had fewer mites than others. It may not be possible to standardize the amount of skin debris collected on an adhesive swab intended for PCR analysis, but for optimal sample collection, we recommend training technicians to systematically sample the mouse pelage by using routine manual restraint and making contact with a large surface area of the mouse, including the interscapular region.

Analyses of body weight, behavior, hematology, serum chemistry analytes, and histopathology demonstrated that neither oral ivermectin treatment nor MI topical therapy had any observable toxicologic effects at the doses and intervals administered. This finding agrees with other studies in which these agents were administered.^{7,77,85} Both ivermectin and moxidectin have been associated with neurologic toxicity in mice, with moxidectin being less toxic than ivermectin; however, we noted no signs of neurotoxicity in our study. Macrocytic lactone toxicity is more pronounced in various mouse strains, particularly those that are deficient in p-glycoprotein/multidrug resistance protein 1, which transports foreign substances across membranes.^{41,51,53,60,84} High doses of imidacloprid can result in behavioral changes, as well as changes in hematologic parameters, liver enzymes, and/or organ pathology in rodents.^{8,46,47,49} Both BUN and liver enzymes (ALP and AST) showed slight differences among the treatment groups, but these changes were not biologically relevant because they were small and within reported ranges for mice.^{56,58,86} In addition, 2 of 10 MI-treated animals had elevated AST values, but these increases cannot be definitively attributed to MI treatment as no other signs of toxicity were observed and 1 mouse had an elevated AST prior to treatment.

Mammals infested with *Demodex* often get secondary bacterial infections.⁶⁹ In dogs, *Staphylococcus pseudintermedius* is commonly associated with demodicosis.⁶⁴ In humans, *S. epidermidis* and *Bacillus* spp. have been implicated in the pathogenesis of *Demodex*-associated rosacea.⁴² We previously reported that

TRP1/TCR mice and NSG mice are highly susceptible to *C. bovis* and *P. pneumotropica* infections.⁸² To minimize colonization with these and other opportunistic bacteria in TRP1/TCR and NSG mice, we instituted various precautions, including pretreatment with enrofloxacin and the provision of husbandry by distinct animal husbandry technicians on a different day than for the remaining colony. Why *P. pneumotropica* was not detected in any of the mice at pretreatment is unknown, given that this bacterium is present in the colony.⁹⁶ Perhaps the parents of the subject mice had recently been treated with antibiotics, which are commonly administered to mice in this colony.⁶⁷ Because we did not isolate *C. bovis*, the implemented precautions appear effective.

Although 8 wk of MI appears safe and economical, this treatment regimen may be impractical in large mouse colonies, because individual mice have to be treated topically as many as 8 times, and effects on reproduction have not been evaluated. We have used topical MI successfully to prophylactically treat small groups of imported mice in quarantine.⁸⁰ However, with the increasing use of PCR assays as a diagnostic tool, many institutions have reduced quarantine periods, and an 8-wk treatment course may be undesirable. Unfortunately, 4 wk of topical MI failed in terms of mite eradication; novel therapeutics including isoxazolines may be more effective. Isoxazolines, including sarolaner, afoxolaner, fluralaner, and lotilaner, have recently been found to have significant acaricidal activity against *Demodex* mites and have been tested experimentally for the treatment of canine demodicosis.^{10,24,88,91} Additional research is warranted to investigate whether isoxazolines can be administered orally, are effective, and are nontoxic in mice.

This study had several limitations and challenges. We used TRP1/TCR mice because they were the first strain we found that was consistently infested with *Demodex* mites; other mouse strains may respond differently to treatment. Evaluating treatment efficacy in a more heavily infested strain would be informative. We used only female TRP1/TCR mice because male TRP1/TCR mice were unavailable; male TRP1/TCR have an MHC-restricted T-cell receptor encoded on the Y chromosome and were needed for experiments and breeding.^{65,67} Although male mice were unavailable, both male and female TRP1/TCR mice previously were demonstrated to have mites, and it is their immune—not their hormonal—status that leads to *Demodex* overgrowth.^{2,18,67} To maintain social housing, animals in the 8-wk trial were not individually housed after treatment, but we believe group housing had a limited effect on the transfer of mites because all 5 ivermectin-treated animals were positive on skin biopsy at 1 wk after treatment. Although mice were randomized among treatment groups, animals from different treatment groups could not be cohoused due to the likelihood of mite transmission between cage mates.

This study revealed that ivermectin administered in feed can significantly reduce mite populations but does not eradicate *Demodex* mites when administered for 8 wk at a dose of 12 ppm. In contrast, weekly topical treatment with moxidectin and imidacloprid (3 and 13 mg/kg, respectively) for 8 wk was safe and eradicated mites from a moderately infested immunocompromised strain. Given these results, we recommend 8 wk of topical MI to eradicate *Demodex* mites, followed by testing animals for mites at 12 wk after treatment. In addition, a commercially available PCR assay was highly useful for diagnosis and assessment of treatment efficacy, but we recommend performing FP+DSS or skin biopsy (or both) as additional tests whenever false-negative results would be problematic. Additional studies are warranted to determine whether other treatment protocols

may be more effective and economical, especially when treating large colonies of laboratory mice.

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