Electroporation has been reported to facilitate naked DNA gene transfer in skeletal muscle, but has also been implicated in the pathogenesis of electrical injuries. To assess the effects of electroporation on gene transfer, mouse quadriceps muscles were injected with the luciferase reporter plasmid VR1255 and electroporated with caliper electrodes. Intramuscular luciferase expression was increased 10- to 70-fold by electroporation, depending on the DNA dose and injection volume used. In the absence of plasmid DNA injection, electroporation of quadriceps muscles resulted in rapid elevations in serum creatine phosphokinase activity, but did not elicit visible muscle damage. However, in muscles injected with plasmid DNA and electroporated, visible lesions consistently developed in the areas proximal to electrode placement when field strengths optimal for gene transfer (300 volts/cm) were applied. The development of muscle lesions was independent of plasmid transgene expression and required the presence of plasmid in the muscle during electroporation. Co-injection of poloxamer 188 (pluronic F68) with VR1255 substantially reduced elevations in serum creatine phosphokinase activity following electroporation, but did not inhibit the development of muscle lesions. In non-electroporated muscles, co-injection of poloxamer 188 increased luciferase expression threefold. Poloxamer 188 may thus constitute a useful excipient for intramuscular delivery of naked DNA.

Key words: electroporation, naked DNA, skeletal muscle, poloxamer 188, pluronic F68
remains to be established. In studies of electrical trauma, electroporation has been implicated as a major mechanism of muscle damage [24]. In isolated skeletal muscle cells, progressively greater transmembrane potentials develop as the strength of external electric fields is increased, leading first to transient, then permanent alterations in membrane permeability [25, 26]. Correspondingly, in intact skeletal muscle, myofiber permeabilization is observed within a relatively narrow range of field strengths, above which irreversible membrane damage occurs [27]. For gene transfer, the extent of muscle damage may depend on the exact pulsing parameters used [15, 28, 29] as well as electrode design [27]. However, current theoretical models of electric field propagation in skeletal muscle suggest that the generation of excessively high field strengths proximal to electrodes may be unavoidable [27], and that localized tissue damage will occur as a result.

Here, we demonstrate that electroporation enhances plasmid reporter gene expression in murine skeletal muscle by 10- to 70-fold, depending on the DNA dose and injection volume used. Further, we show that, under conditions resulting in maximal gene transfer and expression, electroporation results in rapid elevations in serum creatine phosphokinase (CPK) activity and the development of plasmid-dependent muscle lesions. Finally, we show that co-injection of the surfactant poloxamer 188 (p188), a nonionic block co-polymer used in a variety of human drug formulations, reduces electroporation-associated elevations in serum CPK activity and independently augments transgene expression, indicating a possible role for p188 in naked DNA-based gene therapy.

RESULTS

Previous investigations in our laboratory have focused on maximizing intramuscular expression of plasmid DNA...
in Fig. 2. In control muscles, the mean value for intramuscular luciferase expression was 0.87 μg/muscle, with a standard error of the mean (SEM) of ± 0.10 μg/muscle, and the number of muscles (n) was 50. Mean values for the experimental groups (n = 10 muscles) ranged from 0.54 to 1.16 μg/muscle. In electroporated muscles, luciferase expression increased approximately 10-fold to an average of 8.5 ± 1.3 μg/muscle (mean ± SEM, n = 50; P < 0.001), with mean values for the experimental groups ranging from 6.4 to 11.1 μg/muscle. Individual electroporated muscles exhibited expression values as high as 25 μg/muscle. Confirming the results of a previous study, intramuscular expression variability was strongly reduced by electroporation [14]. For the data shown in Fig. 2, the % SEM, representing the SEM divided by the mean for each n = 10 muscles, averaged 23% for control muscles versus 15% for electroporated muscles. This reduction in variability seemed to reflect an absence of low-level expression (Fig. 2).

In the experiments described above, we consistently observed visible damage on the surface of muscles electroporated at field strengths of 300 V/cm or more. The damage was manifested as white spots on or below the muscle surface, generally proximal to the area of electrode placement. Histological analyses of day 7 muscle cross-sections stained with hematoxylin and eosin (see Fig. 3 legend for details) revealed extensive lesions containing necrotic myofibers and heavily populated with infiltrating inflammatory cells (Fig. 3A). The infiltrating cells consisted primarily of mononuclear cells, although polymorphonuclear leukocytes and lymphocytes could also be seen. Typically, remnants of myofibers contained large phagocytic cells and were encircled by fibroblasts (Fig. 3B). In some cases, mineralization was observed in sarcolemmal bundle remnants (Fig. 3C). In non-electroporated control muscles injected with VR1255, smaller lesions containing necrotic myofibers and infiltrating inflammatory cells were present in some of the muscles examined (Fig. 3D). More typically, the control muscles exhibited minor populations of infiltrating cells in the vicinity of the central tendon (Fig. 3E).

Analyses of electroporated muscles from earlier time points revealed damaged myofibrillar bundles and infiltrating polymorphonuclear leukocytes at day 1 (Fig. 3F). By day 3, infiltrating mononuclear cells were also evident. Analyses of electroporated muscles from later time points indicated the lesions observed at day 7 were transient in nature. By day 28, visible lesions were no longer evident, and the regions containing necrotic myofibers and mononuclear infiltrates at day 7 seemed to have undergone regeneration. Within these regions, small foci containing acellular pockets and mineralized remnants of myofibers suggestive of scar tissue could be seen (Fig. 3G). In addition, highly localized lymphocytic infiltrates were present in the vicinity of specific myofibers distributed throughout the muscle (Fig. 3H). Similar localized infiltrates were evident in non-electroporated muscles injected with VR1255. By day 56, sporadic minor foci of acellular pockets and/or mineralization persisted. In general, however, the electroporated muscles were indistinguishable in appearance at day 56 from non-electroporated controls.

To investigate the relationship of electroporation-associated muscle damage to plasmid DNA injection and gene expression, muscles were injected with 50 μl vehicle, with 50 μg of the noncoding plasmid VR1055 in 50 μl vehicle, or with 50 μg of VR1255 in 50 μl vehicle. The muscles were then electroporated as above and harvested at day 7. Visible lesions were evident in muscles injected with VR1255 or VR1055, whereas vehicle-injected muscles had a normal appearance (n ≥ 6 muscles). Histological analysis of the DNA-injected muscles revealed extensive lesions containing necrotic myofibers and heavily populated with infiltrating inflammatory cells. No difference was discernable in the lesions observed in VR1055-injected electroporated muscles (Fig. 3I) versus VR1255-injected electroporated muscles (Fig. 3A). In muscles injected with vehicle alone and electroporated, minor foci of acellular pockets were visible proximal to the areas of electrode placement, but the lesions characteristic of DNA-injected electroporated muscles at day 7 were consistently absent (Fig. 3J). A major histological feature of the vehicle-injected electroporated muscles was the presence of myofibers with centralized nuclei characteristic of regeneration (Fig. 3K).

Enhanced gene transfer is not observed when DNA is injected following electroporation [14]. To determine whether the development of muscle lesions required gene transfer, muscles (n = 20) were injected with 50 μg VR1255...
at 1–2 minutes following electroporation. At day 7, the muscles were harvested and visually inspected for damage. Minimal superficial lesions were evident in 2 muscles, whereas the remaining 18 muscles were indistinguishable from those of naive mice. In muscles processed and assayed for luciferase activity, expression was not statistically different from that of unelectroporated muscles (0.76 ± 0.19 versus 1.13 ± 0.21 μg/muscle, respectively; n = 10, P > 0.05). Histological analysis of the remaining 10 muscles revealed foci of regenerating myofibers proximal to areas of electrode placement, but none of the lesions and inflammatory infiltrates characteristic of muscles injected with DNA before electroporation (Figs. 3L and 3M). In general, the muscles were similar in appearance to vehi-
muscle damage, VR1255 was co-injected with increasing to inhibit the effects of electrical trauma in rat skeletal muscle troporation on gene transfer.

Troporated mice injected with the p-mEPO plasmid showed decreased mEPO expression. To directly quantitate expression of p-mEPO in electroporated mice, hematocrits ranging from 56% to 80% were observed in 19 of 20 mice, reaching mean plateau values of 66–70% within 3 weeks, and remaining at this level through 18 weeks, the longest time point tested.

The absence of elevated hematocrit values in non-electroporated mice injected with the p-mEPO plasmid suggested the reduced injection volume used may have decreased mEPO expression. To directly quantify expression at this injection volume, mice were injected with 10 μg of the luciferase reporter plasmid VR1255 in 10 μl vehicle, and replicate groups were either electroporated at 300 V/cm as above or were not electroporated. Muscles were harvested at day 7 post-injection and assayed for luciferase activity. In control muscles, luciferase expression averaged

cle-injected electroporated muscles.

To assess the short-term effect of electroporation on skeletal muscle, mice were injected and electroporated as above, and serum was collected 2 hours later and assayed for the activity of CPK, an enzymatic marker of myofiber lysis [30]. Serum CPK activity was increased sixfold in electroporated versus non-electroporated mice (P < 0.001; Fig. 4A). Statistically equivalent increases in CPK activity were detected in sera from electroporated mice whether the muscles were injected with VR1255 DNA, with VR1055 DNA, with injection vehicle alone, or were not injected (n = 15 muscles; P > 0.05; data not shown). Thus, in contrast to the muscle lesions observed at day 7, the elevations in serum CPK activity observed at 2 hours following electroporation were not plasmid DNA-dependent. To determine whether gene transfer had occurred within 2 hours, VR1255-injected muscles were harvested and assayed for luciferase expression. In electroporated muscles, expression was increased eightfold relative to non-electroporated muscles (3.72 ng/muscle versus 0.46 ng/muscle, respectively; n = 18, P < .001), indicative of a rapid effect of electroporation on gene transfer.

The nonionic surfactant p188 has been reported to inhibit the effects of electrical trauma in rat skeletal muscle [31]. In an attempt to mitigate electroporation-associated muscle damage, VR1255 was co-injected with increasing concentrations of p188. Muscles were electroporated as above, and serum was collected at 2 hours post-injection and assayed for CPK activity. A dose-dependent decrease in serum CPK activity was detected (Fig. 4A). In mice co-injected with 4% (weight/volume) p188, CPK activity declined 30% relative to controls injected with VR1255 alone (n = 30, P < 0.001). Despite its inhibitory effect on CPK release, p188 co-injection did not appreciably alter the occurrence or extent of electroporation-associated muscle lesions at day 7 (Fig. 3N). The effect of p188 co-injection on intramuscular luciferase expression is shown in Fig. 4B. In electroporated muscles, p188 co-injection did not significantly alter luciferase expression relative to muscles injected with VR1255 alone (8.24 ± 0.67 versus 8.55 ± 0.67 μg/muscle, respectively; n = 50, P > 0.05). However, in non-electroporated muscles, co-injection of 4% p188 increased luciferase expression threefold (2.6 ± 0.55 versus 0.87 ± 0.10 μg/muscle, respectively; n ≥ 30, P < 0.05). Dose-response experiments revealed the effect of p188 on luciferase expression was maximal at this concentration [32].

To examine the effect of electroporation in a therapeutic model, we used a plasmid encoding mouse erythropoietin (p-mEPO). Based on dose-response experiments in which the p-mEPO plasmid was injected in 50 μl vehicle (data not shown), the minimal effective dose of 0.3 μg was chosen. In addition, the volume of injection was reduced to 10 μl to more closely model human and veterinary applications where proportionately smaller injection volumes are used. Mice (n = 20) were injected unilaterally in the quadriceps with 0.3 μg of the p-mEPO plasmid, or an equivalent dose of a plasmid expressing human secreted embryonic alkaline phosphatase (p-SEAP) as a negative control. The injected muscles were electroporated at 300 V/cm as above. For each plasmid, a corresponding group of mice was injected without electroporation. Blood was collected by orbital sinus puncture at various time points, and hematocrits were determined. In the p-mEPO-injected, non-electroporated controls, hematocrits > 55% were not observed, and mean hematocrit values did not differ significantly from those of the p-SEAP negative controls (n = 20 mice; P > 0.05; Fig. 5). However, in the p-mEPO-injected electroporated mice, hematocrits ranging from 56% to 80% were observed in 19 of 20 mice, reaching mean plateau values of 66–70% within 3 weeks, and remaining at this level through 18 weeks, the longest time point tested.

The absence of elevated hematocrit values in non-electroporated mice injected with the p-mEPO plasmid suggested the reduced injection volume used may have decreased mEPO expression. To directly quantify expression at this injection volume, mice were injected with 10 μg of the luciferase reporter plasmid VR1255 in 10 μl vehicle, and replicate groups were either electroporated at 300 V/cm as above or were not electroporated. Muscles were harvested at day 7 post-injection and assayed for luciferase activity. In control muscles, luciferase expression averaged
0.058 ± 0.017 μg/muscle, less than one-third of the expression level obtained with this dose of VR1255 in a 50 μl injection volume (data not shown). However, in electroporated muscles, luciferase expression was greatly elevated, increasing over 70-fold to an average of 4.1 ± 0.64 μg/muscle. To estimate the effect of the reduced dose and injection volume on gene transfer efficiency, the amount of luciferase expression per μg of injected DNA was calculated and compared with that obtained with the 50 μg/50 μl dose and injection volume as calculated from the data presented in Fig. 2. In electroporated muscles, the 10 μg/10 μl dose and injection volume yielded a 2.4-fold increase in the amount of luciferase expression per μg of DNA. In contrast, a 67% decrease was observed in non-electroporated muscles. Thus, the greater response to electroporation achieved with the reduced dose and injection volume reflected alterations in gene transfer efficiency in both electroporated and non-electroporated muscles.

**Discussion**

The initial aim of the studies described here was to assess the ability of electroporation to increase the absolute level of expression attainable in skeletal muscle injected with naked DNA. For this purpose, an optimal dose (50 μg) and injection volume (50 μl) of the luciferase reporter plasmid VR1255 were injected into mouse quadriceps muscle, and the muscles were electroporated with caliper electrodes using empirically optimized parameters for gene transfer (8 × 20 ms pulses at 300 V/cm). Under these conditions, intramuscular luciferase expression was increased 10-fold by electroporation. Based on body mass, the optimal injection volume for a 20-g mouse extrapolates to 150 cc in a 60-kg human, greatly exceeding the standard 1- to 2-cc volumes used for human intramuscular injections. Therefore, the injection volume for mouse quadriceps muscle was reduced 80% to 10 μl, the minimal volume feasible for manual injections, to more closely approximate the effects of smaller injection volumes. Following injection of mouse quadriceps with 10 μg VR1255 in 10 μl vehicle, luciferase expression was increased 70-fold by electroporation, an increment more consistent with the 100-fold or greater responses reported previously [13,14]. The augmented response to electroporation reflected differing effects of the reduced DNA dose and injection volume on the efficiency of gene transfer in control and electroporated muscles. Compared with the 50 μg/50 μl dose and injection volume, gene transfer efficiency decreased 67% in control muscles, and increased over twofold in electroporated muscles. Thus, using injection parameters more representative of injections in larger species, both the efficiency of gene transfer and the magnitude of the response to electroporation were fundamentally altered.

In a previous study, optimal injection volumes exceeding the fluid capacity of the muscle were demonstrated to increase plasmid uptake in mouse tibialis muscle [33]. The increased plasmid uptake was postulated to result from excess intramuscular hydrostatic pressure created by the injection volume. The optimal injection volume (50 μl) produced swelling of the muscle followed by redistribution of the injected DNA to the area of the myotendinous junction. In contrast, a suboptimal injection volume (5 μl) failed to cause visible swelling of the muscle, and produced a more limited dispersion and uptake of the injected DNA in the area proximal to the injection site. In mouse quadriceps muscle, the optimal injection volume of 50 μl represents ~30% of the muscle fluid volume, and results in considerable swelling of the muscle following injection. Moreover, transfection of myofibers proximal to the myotendinous junction occurs shortly after injection of mouse quadriceps muscles with this volume of DNA [34]. In previous electroporation studies, tibialis muscles were injected with 3–15 μg of plasmid DNA, either while surgically exposed [15,29] or using injection volumes less than 50 μl [14,28]. Under these conditions, elevated hydrostatic pressure is less likely to be generated within the injected muscles. Thus, the greater responses to electroporation...
poration reported in these studies, as well as the 70-fold increase achieved in the present study, may reflect a less efficient transfection of non-electroporated control muscles. Conversely, in electroporated muscles, the use of injection volumes less than 50 µl could have positively affected gene transfer by improving retention of the DNA within the electroporated area of the muscle. Additionally, it is possible that the use of lower doses or concentrations of DNA may reduce the extent of muscle damage.

In the experiments shown here, we have presented evidence that most of the muscle damage arising from electroporation is plasmid DNA-dependent. In muscles injected with plasmid DNA and electroporated, lesions containing necrotic myofibers and heavily populated with inflammatory cells were evident at day 7, while in muscles injected with vehicle and electroporated, visible lesions were not observed and foci of regenerating myofibers were evident at day 7. In contrast to the results at day 7, statistically equivalent elevations in serum CPK activity were detected within 2 hours of electroporation, regardless of whether muscles were injected with DNA, with vehicle, or were not injected. These results imply that (1) electroporation causes equivalent permeabilization of myofibers in the presence or absence of DNA; (2) in the absence of plasmid DNA, electroporation results in limited muscle damage characterized by an absence of inflammatory infiltrates and a rapid regenerative response; and (3) in the presence of plasmid DNA, electroporation results in extensive muscle damage characterized by myofiber necrosis, inflammation, and the development of muscle lesions. Notably, in muscles electroporated before the injection of DNA, muscle lesions did not develop, and gene transfer was not significantly enhanced. Collectively, these results show that the development of electroporation-associated muscle lesions requires DNA transfer.

Injection of muscles with plasmid DNA has been reported to elicit infiltration of mononuclear cells and lymphocytes due to the presence of hypomethylated CpG residues common to DNA of bacterial origin [35]. The proinflammatory response seems to involve specific intracellular receptors for CpG sequence elements present in B cells, monocyte/macrophages, and dendritic cells, all capable of spontaneously taking up exogenous DNA [36]. Infiltrates similar in character to those elicited by CpG residues were observed in the muscle lesions shown in Fig. 3, suggesting a possible role for CpG-based proinflammatory responses in the development of these lesions. However, direct responses of myofibers to stimulatory CpG residues characteristic of DNA may reduce the extent of muscle damage.

FIG. 5. Effect of electroporation on hematocrit following intramuscular injection of plasmid DNA encoding murine erythropoietin. Quadriceps muscles of BALB/c mice were injected unilaterally with 0.3 µg of plasmid DNA encoding murine erythropoietin (p-mEPO) or a negative control plasmid encoding human secreted embryonic alkaline phosphatase (p-SEAP) in 10 µl sodium phosphate vehicle with (+EP) or without (-EP) electroporation. At various time points, blood was collected and hematocrits were determined. Values shown are averages ± SEM; n = 20 mice.

Intravenous administration of P188, an 8.6-kDa non-ionic surfactant, has been reported to inhibit the effects of electrical trauma in rat skeletal muscle [31]. In the present study, intramuscular injection of P188 with plasmid DNA was investigated as a means of reducing electroporation-associated myofiber lysis. Following electroporation, average decreases in serum CPK activity of approximately 30% were observed in mice co-injected with P188 relative to mice injected with plasmid DNA alone. Membrane defects in myofibers have been demonstrated following the delivery of electrical pulses to skeletal muscle [37], and P188 has been shown to promote membrane resealing in electroperturbed skeletal muscle cells in vitro [31]. Therefore, the decrease in CPK activity described above most likely reflects P188-enhanced membrane resealing in permeabilized myofibers. These experiments demonstrate that P188 co-injection may be useful in counteracting the effects of electrical trauma in electroporated muscles.

In the absence of electroporation, a threefold increase in intramuscular luciferase expression was observed following co-injection of VR1255 with 4% P188. This result extends recent findings using a combination of two poloxamers [38], and more generally confirms the ability of poloxamers to enhance plasmid DNA-based gene expression. The
mechanism(s) whereby poloxamers enhance expression is unknown, but may include effects on DNA uptake, dispersion, or resealing of membrane defects in myofibers [38–40]. Poloxamer 188 is listed in the US Pharmacopeia as an inactivating excipient. It is used pharmaceutically in a variety of applications [41], and thus exhibits a favorable safety profile for use as an excipient for naked DNA vaccines and therapeutics. A more thorough investigation of the effects of p188 and other poloxamers on naked DNA gene expression and vaccine potency is ongoing in our laboratory [32].

We have demonstrated that co-injection of p188 with plasmid DNA inhibits systemic elevations in CPK activity due to electroporation and independently augments plasmid transgene expression. Furthermore, using injection parameters optimized for mouse quadriceps muscle, we have shown that electroporation increases plasmid transgene expression 10-fold, but also results in the development of plasmid DNA-dependent muscle lesions. We are cognizant of the likelihood that, due to the large volume of injection vehicle used and the large volume of muscle tissue electroporated, our results may both underestimate the effect of electroporation on gene transfer and overestimate the extent of associated muscle damage when extrapolated to larger species. Nonetheless, by providing new insight into the effects on electroporation on skeletal muscle, our results also provide an experimental basis to further stimulate development of this promising technique for human intramuscular gene transfer.

**MATERIALS AND METHODS**

**Reagents.** Poloxamer 188 (Pluracare F68, Prill), a block copolymer of the general formula poly(oxyethylene)₉₉–poly(oxypropylene)₉₉–poly(oxyethylene)₉₉, was obtained from BASF Corp. (Mt. Olive, NJ). A stock solution of 10% (weight/volume) p188 was prepared in 150 mM sodium phosphate, pH 7.2, and stored at 4°C before use.

**Plasmid DNA.** The luciferase expression plasmid VR1255 comprises the human cytomegalovirus immediate early gene enhancer/promoter and intron A, the Escherichia coli origin of replication, and the kanamycin resistance gene [11]. The noncoding plasmid VR1055 contains a multiple cloning site in place of the luc+ gene and is otherwise identical to VR1255 [5]. Plasmids p-mEPO and p-SEAP comprise the cDNAs for murine erythropoietin or human secreted embryonic alkaline phosphatase, respectively, subcloned in the VR1012 plasmid backbone [12]. Plasmid DNA for injection was prepared by CsCl/ethidium bromide gradient centrifugation as described [42]. Quality control assays were performed to ensure that contaminating endotoxin levels were ≤0.005 endotoxin units/µg DNA, and that the plasmid was predominately in the supercoiled form.

**Intramuscular injections.** Female BALB/c mice, aged 8–10 weeks, were anesthetized by intraperitoneal injection of 100 µl ketamine/xylazine cocktail (87 µg/g ketamine, 13 µg/g xylazine in saline) plus inhalant Metofane. Mice were deeply anesthetized with Metofane, blood was collected by cardiac puncture, and the mice were euthanized by cervical dislocation. Serum was prepared and stored at 4°C for up to 5 d. For assay, serum was diluted 1:10 in distilled water and assayed using Sigma kit 520C (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions, except the assay volumes were scaled down to allow the use of microtiter plates. Reactions were incubated at 37°C for 30 min, stopped by the addition of p-hydroxymercuribenzoate, and the free creatine product was measured colorimetrically by absorbance at 490 nm. A standard curve was generated using the creatine standard supplied with the kit, and the results were expressed as International Units/Liter (IU/L, corresponding to µmol creatine formed/min/L) using the conversion factor supplied by Sigma.

** Luciferase assays.** Mice were sacrificed by sodium pentobarbital overdose. The entire quadriceps muscle group was removed surgically, frozen immediately on dry ice, and stored at –80°C. Muscle extractions and assays were performed as described [43]. For extrapolation of intramuscular luciferase protein concentrations, a purified firefly luciferase standard (Analytical Luminescence Labs, Ann Arbor, MI) was used to generate a standard curve. Under the conditions of the assay, 33.3 pg of the standard generated an average of 5.09 × 10³ relative light units in a Dynatech Model 2250 microplate luminometer (Dynatech, Chantilly, VA).

**Histopathological analysis.** Quadriceps muscles were harvested as above and fixed in 4% (weight/volume) paraformaldehyde in phosphate-buffered saline at 4°C overnight. The muscles were embedded in paraffin and 8 µm cross-sections were cut at 50 µm intervals from the center of the muscle (that is, midway between the knee and hip) extending in both directions. The sections were stained with hematoxylin and eosin by standard procedures. Analysis of tissue sections for characterization of muscle pathology and identification of infiltrating cell types was performed unblinded by a veterinary pathologist (Sheldon Diamond, Pathology Associates International, San Diego, CA).

**Statistical analysis.** All statistical comparisons were carried out using the non-parametric Mann-Whitney rank sum test (SigmaStat version 2.03, Jandel Scientific Software, San Rafael, CA). This test was used because it does not require a normal distribution of data values about the mean, and may be used to compare experimental groups differing in size.

**Hematocrit determinations.** Mice were lightly anesthetized with inhalant Metofane, and blood was collected by orbital sinus puncture into heparinized capillary tubes. Hematocrits were determined using a HemataSTAT II centrifuge (S.T.I., Altamante Springs, FL) according to the manufacturer’s instructions.

**Animal care and use.** Animal procedures and husbandry were compliant with the ‘Guide for the Use and Care of Laboratory Animals’ (National Academy Press, Washington, DC, 1996), and were additionally subject to approval by the Institutional Animal Care and Use Committee.
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