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DNase Expression Allows the Pathogen Group A *Streptococcus* to Escape Killing in Neutrophil Extracellular Traps

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Summary

The innate immune response plays a crucial role in satisfactory host resolution of bacterial infection. In response to chemotactic signals, neutrophils are early responding cells that migrate in large numbers to sites of infection. The recent discovery of secreted neutrophil extracellular traps (NETs) composed of DNA and histones opened a novel dimension in our understanding of the microbial killing capacity of these specialized leukocytes. M1 serotype strains of the pathogen Group A *Streptococcus* (GAS) are associated with invasive infections including necrotizing fasciitis (NF) and express a potent DNase (Sda1). Here we apply a molecular genetic approach of allelic replacement mutagenesis, single gene complementation, and heterologous expression to demonstrate that DNase Sda1 is both necessary and sufficient to promote GAS neutrophil resistance and virulence in a murine model of NF. Live fluorescent microscopic cell imaging and histopathological analysis are used to establish for the first time a direct linkage between NET degradation and bacterial pathogenicity. Inhibition of GAS DNase activity with G-actin enhanced neutrophil clearance of the pathogen *in vitro* and reduced virulence *in vivo*. The results demonstrate a significant role for NETs in neutrophil-mediated innate immunity, and at the same time identify a novel therapeutic target against invasive GAS infection.

Results and Discussion

Mutagenesis, Complementation, and Heterologous Expression of GAS *sda1*

Neutrophil extracellular traps (NETs) are composed of DNA and contain chromatin and neutrophil granule components, which act to capture and kill surrounding bacteria independent of phagocytic uptake [1]. The discovery of NETs and their clear contribution to neutrophil innate immune function raises an interesting question: have certain bacterial pathogens evolved mechanisms to escape NETs in order to enhance their survival within

the host? We hypothesized that DNase activity contributed to virulence of invasive GAS strains by allowing the organism to escape NET-mediated killing. A well-recognized phenotype shared by all strains of GAS is the elaboration of one or more extracellular deoxyribonucleases (DNases) [2, 3]. Long enigmatic, an overall contribution of DNase production to GAS virulence was recently demonstrated in the studies of Sumbly et al. [4]. Allelic replacement mutagenesis of wt serotype M1 GAS strain 5448 targeted the gene encoding Sda1 (see Figure S1 in the Supplemental Data available with this article online). Sda1 was originally discovered on proteomic analysis of a globally disseminated invasive M1T1 GAS clone [5] and encodes the principal extracellular DNase activity [4]. By agarose gel assay, the M1ΔSda1 mutant demonstrated a clear decrease in DNase activity (Figure 1A). DNase activity could be restored to wt levels in the mutant by transformation with a plasmid (pSda1) bearing the cloned *sda1* gene (Figure 1A), thus excluding polar effects of mutagenesis. Moreover, the M1ΔSda1 mutant was equivalent to wt in expression of secreted cysteine protease SpeB and surface bound M1 protein (Figure S2A). Wild-type and ΔSda1 mutant also possessed equal hemolytic activities (Figure S2B). Together, these studies exclude pleiotropic mutations on certain virulence factors that promote GAS phagocyte resistance. Finally, clear increases in extracellular DNase activity could be appreciated in a noninvasive serotype M49 isolate of GAS as well as the nonpathogenic gram-positive bacterium *Lactococcus lactis* when each was transformed with pSda1 (Figure 1A). A panel of bacterial reagents was thus generated to test the role of DNase Sda1 in pathogenesis by means of both loss- and gain-of-function analyses.

GAS DNase Promotes Bacterial Survival in Neutrophil- and Blood-Killing Assays

To test a potential role of DNase Sda1 in GAS resistance to innate immune clearance, coinubation experiments were performed with bacteria and freshly isolated human neutrophils. The wt M1 GAS strain was found to be significantly more resistant to neutrophil killing than the isogenic M1ΔSda1 mutant at a number of different bacterial inocula and incubation times (Figures 1B and 1C). Focusing specifically on extracellular bactericidal mechanisms including NETs, cytochalasin D (CytD) was added to block phagocytotic uptake [1], resulting in a further exaggeration of the survival deficit of the M1ΔSda1 mutant (Figure 1D). Expression of Sda1 in a noninvasive serotype M49 GAS strain conferred enhanced resistance to neutrophil extracellular killing, a finding echoed in nonpathogenic *Lactococcus lactis* transformed with pSda1 (Figure 1D). Taken together, these data demonstrate that Sda1-mediated DNase activity is both necessary and sufficient to promote bacterial resistance to neutrophil killing. Elimination of Sda1 also rendered GAS more susceptible to clearance in whole mouse blood (Figure 1E),

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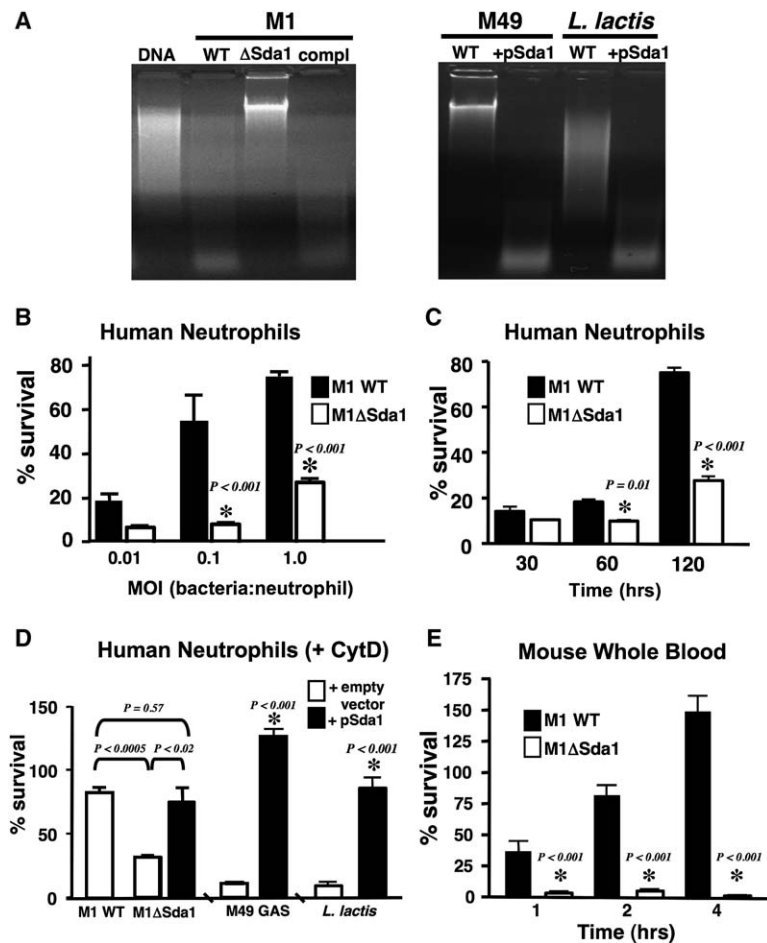


Figure 1. Expression of DNase Sda1 Promotes Resistance to Neutrophil Killing

(A) Activity of bacterial supernatants in degrading calf thymus DNA by agarose gel electrophoresis. Strains tested were wild-type (wt) M1 GAS, knockout mutant Δ Sda1, complemented knockout mutant transformed with pSda1 (compl), GAS M49 wt, M49 + pSda1, *Lactococcus lactis* wt, and *L. lactis* + pSda1.

(B and C) Survival of wt M1 GAS and isogenic Δ Sda1 mutant in coculture with human neutrophils (B) at 2 hr at various multiplicity of infection (moi) or (C) over time (moi = 1.0).

(D) Effect of *sda1* expression on GAS M1 (moi 1.0), GAS M49 (moi = 0.5), and *Lactococcus lactis* (moi = 1.0) survival of extracellular killing by human neutrophils (10 μ g/ml of the cytochalasin D used to block phagocytosis). Also shown is survival of GAS M1 Δ Sda1 complemented with pSda1 to restore DNase activity. (E) Survival of wt M1 GAS and isogenic Δ Sda1 mutant in mouse whole blood. Values reported are means \pm SEM. Results shown are representative of at least three experiments repeated with similar results.

suggesting that DNase expression could contribute to phagocyte resistance in more complex host tissue environments.

DNase Sda1 Promotes Virulence in a Mouse Model of GAS NF

To examine the contribution of the Sda1 DNase to virulence in vivo, we employed a murine model of GAS NF. Mice challenged subcutaneously with the wt M1 GAS strain developed significantly larger lesions than those challenged with an identical dose of the M1 Δ Sda1 mutant (Figures 2A and 2B). Upon sacrifice and skin biopsy 5 days postinfection, the lesions of animals infected with the wt harbored approximately 100-fold greater concentrations of viable GAS (CFU/gm) than lesions from mice infected with the M1 Δ Sda1 strain (Figure 2C). Corroborating findings were provided in challenge experiments comparing the M49 GAS strain (low baseline DNase expression) to its counterpart transformed with pSda1. Overexpression of Sda1 in the GAS M49 background produced a significant increase in skin lesion size and bacterial proliferation in vivo (Figures 2A–2C). Thus, genetic manipulation of GAS DNase expression altered virulence potential in vivo in a fashion that mirrored the observed effects on resistance to neutrophil extracellular killing ex vivo.

GAS DNase Sda1 Promotes Degradation of NETs

We hypothesized that degradation of the DNA framework of NETs could represent the underlying mechanism to explain the contribution of Sda1 to GAS resistance to neutrophil killing and virulence potential. To examine this interaction, we developed assays for real-time visualization of NET formation by freshly isolated human neutrophils via the Sytox family (Molecular Probes) of fluorescent DNA stains. Because these stains do not penetrate cell membranes effectively, fluorescent DNA staining is specific to secreted extracellular DNA (e.g., NETs) or to nuclear DNA of dead cells. By using this live-cell imaging technique, we were able to observe distinct NET formation by isolated neutrophils within 10 min of isolation. The rapid appearance of NETs supported the conclusions of Brinkman et al. who concluded that NET phenomenon is an active process independent of necrosis or apoptosis by using differential staining of formalin-fixed cells [1]. Over the course of 15 min, the wt M1 GAS strain was able to effect a dose-dependent elimination of NETs, while significant quantities of NETs persisted in companion assays by means of the M1 Δ Sda1 mutant. For gain-of-function analysis, we tested *L. lactis*, which lacked the ability to eliminate NETs upon coincubation with human neutrophils. Heterologous expression of GAS Sda1 in *L. lactis* allowed the transformed bacteria to degrade NETs (Figure 3A). These studies revealed DNase Sda1 to be necessary and sufficient for

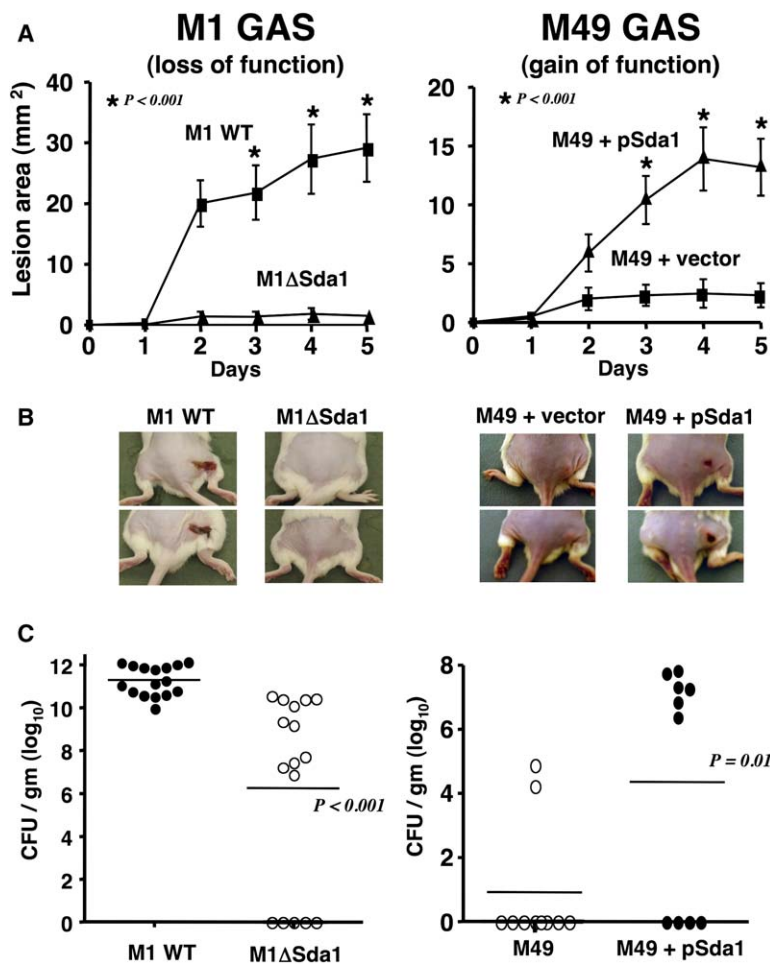


Figure 2. GAS DNase *sda1* Contributes to Bacterial Virulence in a Mouse Model of Necrotizing Fasciitis

(A) CD1 mice were injected subcutaneously in the flank with 1×10^6 CFU wild-type (wt) GAS M1 or M1ΔSda1 ($n = 15$ per group), or with 1×10^7 CFU wt GAS M49 (plasmid control) or M49 + pSda1 ($n = 10$ per group). The size of necrotic skin lesions was measured daily for 5 days. Presence of Sda1 was correlated to increased virulence potential. Values reported are mean \pm SEM.

(B) Representative photographs of skin lesions in mice from (A).

(C) Number of bacteria recovered after 5 days from the site of injection of 1×10^6 CFU of GAS M1 wt or M1ΔSda1 mutant, or M49 (plasmid control) and M49 + pSda1. Data were log₁₀ transformed; bar indicates mean.

effective bacterial degradation of NETs and correlated directly with the respective ability of the various bacterial strains to survive extracellular killing when coincubated with neutrophils (Figure 1D).

Additional studies were performed to clarify whether Sda1-mediated degradation of NETs per se represented a key pathogenic phenotype in the early stages of GAS infection in vivo. Biopsies from mice infected with the wt M1 GAS or the M1ΔSda1 mutant at 4 hr post subcutaneous injection revealed similar levels of neutrophil infiltration and early abscess formation (Figure S3A). The number of recruited leukocytes stimulated by the wt strain and M1ΔSda1 mutant was also similar when the bacteria were implanted into subcutaneous tissue cages for accurate serial sampling over 72 hr (Figure S3B). Purulent exudates from the early stages of abscess formation in the subcutaneous challenge model (4 hr) were collected for DNA staining to visualize NET formation in vivo. In each of four mice infected with wt M1 GAS in which clear exudates of intact neutrophils were collected, no NETs could be visualized (Figure 3B). In contrast, NETs were common in the purulent exudates obtained from a group of mice challenged with the identical inoculum of the M1ΔSda1 mutant (Figure 3B). These findings support the mechanistic linkage of GAS Sda1 DNase-mediated enhancement of bacterial survival and NET degradation ex vivo (Figures 1B–1E and

3A) with enhanced bacterial virulence potential in vivo (Figure 2).

A DNase Inhibitor Increases GAS Sensitivity to Neutrophil Killing

The identification of GAS DNase Sda1 degradation of NETs as a critical element in the pathogen's ability to resist extracellular killing suggested that neutralization of this virulence phenotype could render the organism more susceptible to host neutrophil clearance. As a proof of concept for pharmacological targeting of GAS DNase production, we performed total and extracellular (+ CytD) neutrophil killing assays with wt and M1ΔSda1 mutant GAS in the presence and absence of G-actin, a known inhibitor of DNase I activity [6, 7]. Addition of the DNase inhibitor produced a marked decrease in the ability of GAS wt M1 strain to resist neutrophil killing, rendering it equally susceptible to clearance as the GAS M1ΔSda1 mutant (Figure 4A). This finding reinforced the link between bacterial DNase activity and escape from neutrophil extracellular killing. Further, we observed that the effect of DNase inhibition could be extended in vivo. Mice were challenged simultaneously with an equal inoculum of GAS each flank. In one injection site, the DNase inhibitor G-actin was simultaneously introduced, while the opposite received vehicle alone, allowing each animal to serve as its own control.

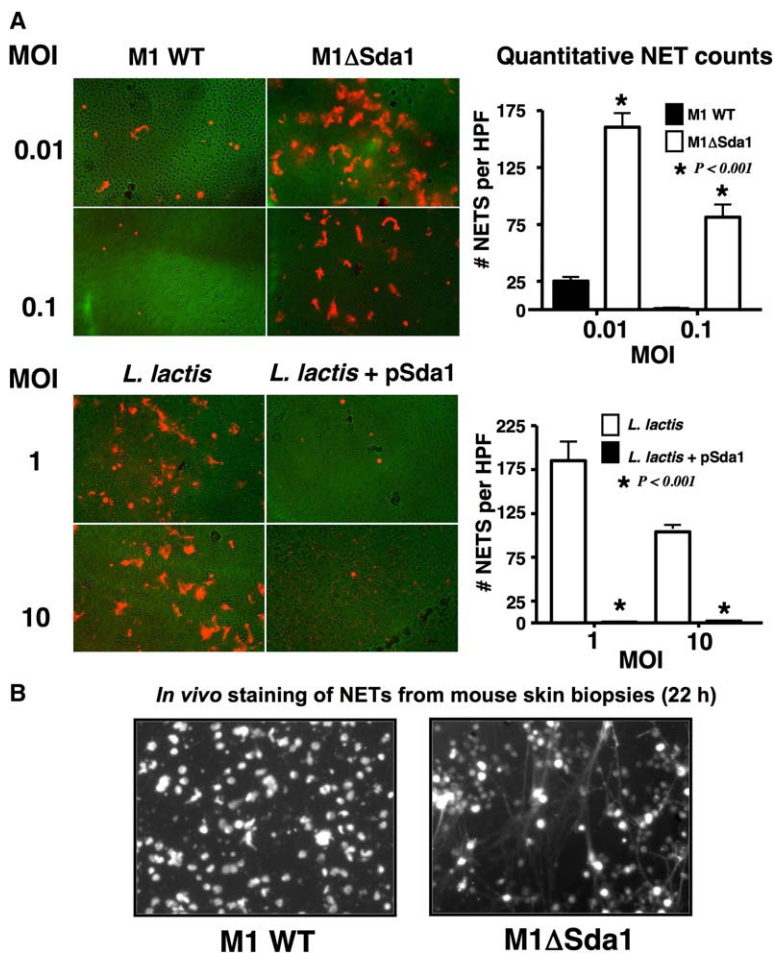


Figure 3. GAS DNase Sda1 Degrades NETs In Vitro and In Vivo

(A) Fluorescent images of DNA NETs stained with Sytox Orange overlaid with a brightfield image of human neutrophils. Wild-type M1 GAS or M1ΔSda1, and *Lactococcus lactis* (plasmid control) and *L. lactis* + pSda1 on a plasmid (pSda1) were cocultured with human neutrophils. NETs were stained without fixation after 15 min, appearing bright orange. Presence of Sda1 was correlated to elimination of NETs. For quantitation, NETs were enumerated for each treatment by counting transects after staining for NETs; a NET was defined as a discrete area of bright orange fluorescence larger in size than a neutrophil. (B) Representative grayscale image of NETs present in abscess exudates 22 hr after subcutaneous injection of wt M1 GAS or M1ΔSda1 mutant. Abscess exudates were stained with Sytox orange and visualized fluorescently. NETs were clearly visualized in 4/4 mice injected with M1ΔSda1 mutant and in 0/4 mice injected with wt M1 GAS.

We observed that necrotic lesions on the side treated with G-actin were markedly smaller than the untreated controls (Figure 4B).

Conclusions

Neutrophils effectively kill bacteria through a variety of mechanisms involving phagocytosis and/or degranulation, including proteolytic enzymes, antimicrobial peptides, and reactive oxygen and nitrogen species [8]. The landmark study of Brinkmann et al. [1] describing NETs revealed a novel mechanism by which host neutrophils extend their ability to defend against invading microbes. The authors speculated that within NETs, antimicrobial peptides and proteases are concentrated at high levels to effect bacterial killing. The present work provides the first demonstration of a pathogenic bacterium facilitating its escape from neutrophil killing by degradation of NETs, providing complementation and heterologous expression studies that establish molecular Koch's postulates for Sda1 as a virulence factor.

Over the last three decades, a resurgence of severe invasive forms of GAS infection is well documented, including the life-threatening syndromes of necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) [9, 10]. All GAS strains express one or more DNase genes [2, 3, 11], and it is likely that DNase contributes in concert with established virulence factors such

as M protein, hyaluronic acid capsule, and streptolysin S to the overall phagocyte resistance of the pathogen. It is possible that the unusual potency of phage-encoded Sda1 [4, 5] contributes in an important fashion to the strong epidemiologic association of clonal serotype M1 strains with NF and other severe invasive forms of GAS infections.

We have provided evidence that the marked virulence attenuation of GAS *sda1* mutants can be correlated to degradation of NETs in vitro and in vivo. As a corollary to this observation, the phenomenon of NET formation demonstrated in vitro by Brinkman et al. [1] can now be appreciated to represent an essential component of host innate immunity in vivo. As elaboration of DNases has been documented in several other bacterial species of clinical significance [2, 12, 13], future studies can be directed to establish whether degradation and escape from NETs can represent a more broadly generalized mechanism of bacterial virulence. In the case of GAS, our studies indicate that drugs designed to neutralize DNase activity and preserve host NET integrity could represent a novel adjunctive therapy to antibiotic and surgical management.

Supplemental Data

Supplemental Data include three figures and Supplemental Experimental Procedures and are available with this article online at <http://www.current-biology.com/cgi/content/full/16/4/DC1/>.

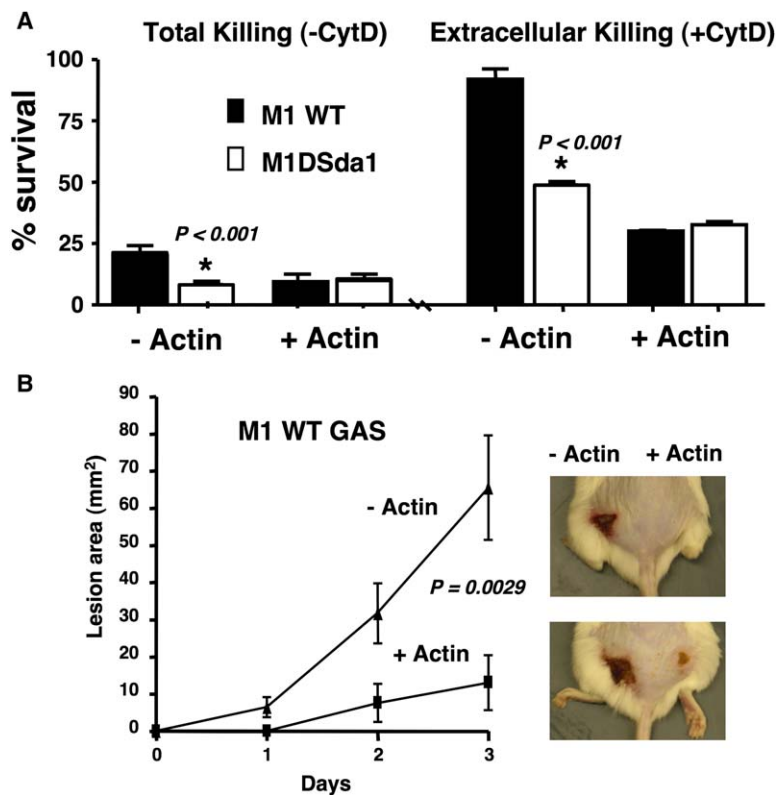


Figure 4. GAS Sda1 Promotion of GAS Survival Can Be Reversed by a DNase Inhibitor

(A) The DNase inhibitor G-actin (100 $\mu\text{g/ml}$) reduces wt M1 GAS survival in coculture with human neutrophils to levels seen with the isogenic M1 Δsda1 mutant, both with and without 10 $\mu\text{g/ml}$ of cytochalasin D to inhibit phagocytosis (moi = 0.01, 2 hr). Values reported are mean + SEM. Results shown are representative of at least three experiments with similar results.

(B) CD1 mice were injected subcutaneously in both flanks with $1-2 \times 10^7$ CFU wild-type (wt) GAS M1 with (right flank) or without (left flank) addition of 165 μg of G-actin (n = 11). The size of necrotic skin lesions was measured daily for 3 days. Injection of actin significantly reduced lesion size (p = 0.0029). Values reported are mean \pm SEM. Photos of two representative animals are shown.

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