High- and ultrahigh-field magnetic resonance imaging of naïve, injured and scarred vocal fold mucosae in rats

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ABSTRACT
Subepithelial changes to the vocal fold mucosa, such as fibrosis, are difficult to identify using visual assessment of the tissue surface. Moreover, without suspicion of neoplasm, mucosal biopsy is not a viable clinical option, as it carries its own risk of iatrogenic injury and scar formation. Given these challenges, we assessed the ability of high- (4.7 T) and ultrahigh-field (9.4 T) magnetic resonance imaging to resolve key vocal fold subepithelial tissue structures in the rat, an important and widely used preclinical model in vocal fold biology. We conducted serial in vivo and ex vivo imaging, evaluated an array of acquisition sequences and contrast agents, and successfully resolved key anatomic features of naïve, acutely injured, and chronically scarred vocal fold mucosae on the ex vivo scans. Naïve lamina propria was hypointense on T1-weighted imaging with gadobenate dimeglumine contrast enhancement, whereas chronic scar was characterized by increased lamina propria T1 signal intensity and mucosal volume. Acutely injured mucosa was hypointense on T2-weighted imaging; lesion volume steadily increased, peaked at 5 days post-injury, and then decreased, consistent with the physiology of acute, followed by subacute, hemorrhage and associated changes in the magnetic state of hemoglobin and its degradation products. Intravenous administration of superparamagnetic iron oxide conferred no T2 contrast enhancement during the acute injury period. These findings confirm that magnetic resonance imaging can resolve anatomic substructures within naïve vocal fold mucosa, qualitative and quantitative features of acute injury, and the presence of chronic scar.

KEY WORDS: Fibrosis, Hemorrhage, Larynx, MRI, Tissue repair, Voice, Wound healing

INTRODUCTION
The vocal fold mucosae are a pair of biomechanically exquisite, voice-generating tissues housed in the larynx. Clinically, vocal fold mucosal integrity is evaluated using direct or indirect laryngoscopy (Rosen and Murry, 2000; Sulica, 2013). Epithelial lesions can be identified visually; however, subepithelial lesions can be difficult to differentiate based on external appearance alone and so are typically inferred from their impact on vocal fold oscillation during voicing (Rosen et al., 2012). This is particularly true in the case of vocal fold scar, which does not alter the mucosal edge contour to the extent of other benign subepithelial lesions (Dailey and Ford, 2006). Pathological diagnosis using mucosal biopsy carries a risk of iatrogenic injury, scar formation and chronic dysphonia, and so is generally reserved for cases involving clinical suspicion of a malignant neoplasm. Consequently, most subepithelial lesions are not definitively diagnosed until the time of surgical resection and pathology readout. There is therefore a need for improved nondestructive assessment of the vocal fold mucosae, to assist with provisional diagnosis, treatment planning and disease monitoring.

A number of nondestructive imaging modalities have been proposed in an attempt to better evaluate the vocal fold mucosa in situ. Optical coherence tomography (OCT) and high-frequency (>30 kHz) ultrasound provide high-resolution, cross-sectional imaging of tissues and have been used to evaluate naïve, pathologic and surgically manipulated vocal fold mucosae in preclinical models and human patients (Burns et al., 2011, 2009; Coughlan et al., 2016; Huang et al., 2007; Walsh et al., 2008; Wong et al., 2005). Imaging data are available in real time; however, with the exception of long-range OCT (Coughlan et al., 2016; Vokes et al., 2008), these techniques require endolaryngeal placement of an imaging probe used in contact or near-contact mode, have limited depth penetration and do not provide full anatomic context for the region of interest. Magnetic resonance imaging (MRI) is an alternative technology that allows high-resolution, high-contrast imaging of whole tissues. Unlike other whole-specimen imaging techniques such as X-ray and computed tomography, MRI does not deliver ionizing radiation. It does not require placement of an imaging probe, is not limited to cross-sectional imaging and can be used to acquire three-dimensional data. Clinical MRI is generally performed using a field strength of 1.5-3.0 T; however, preclinical MR instruments are commercially available with field strengths as high as 21.1 T (Scheplin et al., 2010; Sharma, 2009; Sharma and Sharma, 2011), providing spatial resolution comparable with the ~10-50 μm reported for OCT and high-frequency ultrasound. A previous report of ultrahigh-field (11.7 T) imaging of ex vivo ferret and canine larynges showed clear identification of basic vocal fold sub-structures, experimentally induced scar, and injected biomaterials at 39 μm²/pixel resolution (Herrera et al., 2009). This proof-of-concept study demonstrated the potential of MRI for the nondestructive characterization of vocal fold subepithelial tissue changes.

Here, to expand on this previous work, we assessed the ability of high- and ultrahigh-field MRI to resolve key vocal fold tissue structures in the rat; an important and widely used preclinical model...
in vocal fold biology (Riede et al., 2011; Tateya et al., 2006; Welham et al., 2015). We conducted serial in vivo and ex vivo imaging, evaluated an array of acquisition sequences and contrast agents, and successfully characterized features of both acute vocal fold injury and chronic vocal fold scar.

RESULTS
MRI of the naïve rat larynx
To our knowledge, despite the availability of human and large animal data (Chen et al., 2012; Herrera et al., 2009), there are no previous reports of MRI of the rat larynx. We therefore began by imaging naïve rats in vivo and naïve rat larynges ex vivo to evaluate the ability of MRI to resolve key anatomic structures at 4.7 and 9.4 T. T1-weighted (T1W) in vivo imaging of the rat neck with intravenous gadobenate dimeglumine (Gd) contrast enhancement provided clear identification of the glottis and some cartilaginous structures at 273 µm/voxel resolution, but did not resolve individual cartilages, muscles or sub-structures within the vocal fold mucosa (Fig. 1A-B). Overnight (~6 h) T1W imaging of ex vivo naïve larynges following 10 days of Gd immersion contrast enhancement allowed identification of hyperintense vocal fold mucosae, individual intrinsic laryngeal muscles and hypointense laryngeal cartilages (Fig. 1C). These structures were identified at 41 µm/voxel resolution; we obtained comparable resolution of key laryngeal sub-structures with 10 min T1W scans at 9.4 T (Fig. 1D). The acquisition of three-dimensional data allowed precise volume rendering of all laryngeal structures (Fig. 1E).

Evaluation of acute vocal fold injury with intravenous SPIO
Vocal fold mucosal injury in the rat model results in peak cellular infiltration at 5 days post-injury (Ling et al., 2010a). This infiltrating population includes monocyte lineage cells, such as fibrocytes and macrophages (Ling et al., 2010b). As proinflammatory macrophages are known to engage in iron uptake and sequestration (Cairo et al., 2011), and because paramagnetic iron causes shortening of T2 relaxation time on MRI (Chen et al., 1999), we evaluated whether the intravenous delivery of superparamagnetic iron oxide (SPIO) nanoparticles could enhance MRI contrast of the acutely injured vocal fold mucosa. This approach has been successfully used to study macrophage infiltration of both central and peripheral nervous system injuries in experimental models (Bendszus and Stoll, 2003; Kleinschnitz et al., 2003; Stoll et al., 2004), as well as identification of liver and spleen lesions on clinical MRI (as most circulating SPIO is eventually phagocytized by Kupffer cells in the liver and red pulp macrophages in the spleen) (Chen et al., 1999; Schulmann-Giampieri, 1993).

We created unilateral vocal fold mucosal injuries, injected intravenous SPIO 4 days post-injury and performed in vivo followed by ex vivo imaging 5 days post-injury. Non-SPIO-treated rats served as controls. Abdominal scans showed liver hypointensity on T2W and T2*W images following SPIO administration, confirming successful nanoparticle migration and uptake by Kupffer cells in vivo (Fig. 2A). Despite this evidence of cell-mediated modulation of liver signal intensity, we were unable to resolve the vocal fold lesions in vivo, owing to insufficient imaging resolution (Fig. 2A). Follow-up T2W imaging of the explanted larynges ex vivo resulted in clear identification of the unilateral lesions as hypointense tissue regions, irrespective of the presence or absence of SPIO (Fig. 2B). SPIO contrast enhancement was associated with larger lesion volumes in certain cases (Fig. 2B-C); however, quantitative analysis of lesion volumes showed no overall advantage with SPIO (P>0.01; Fig. 2D). We identified residual hemorrhage and hemosiderin on hematoxylin and eosin (H&E) staining, ferric iron on Prussian Blue staining, and CD68+ macrophages on immunostaining (Fig. 2E). These features were present in both the presence and absence of SPIO.

Characterization of the acute vocal fold injury time course
Given that intravenous SPIO conferred no benefit during ex vivo T2W imaging of acute vocal fold injury at 5 days post-injury, we proceeded to characterize the acute injury time course without SPIO. We created unilateral injuries as described above and performed ex vivo imaging at 1, 3, 5 and 7 days post-injury. The hypointense vocal fold lesions were clearly identified with T2W imaging at each time point (Fig. 3A). Lesion volume steadily increased over the first 5 days, peaked at day 5, and decreased on day 7 post-injury (P<0.01; Fig. 3B-C). We acquired additional T2*W images 1 day post-injury that also showed tissue hypointensity at the lesion site (Fig. 3D), consistent with acute hemorrhage (Bradley, 1993). Using histology, we confirmed the presence of acute, and then resolving, hemorrhage over the experimental time course (Fig. 3E). Ferric iron was first detected...
unilateral vocal fold mucosal injuries and performed ability to resolve vocal fold scar tissue with MRI, we created

imaging sequence following Gd immersion (Fig. 4A–C). Post-scan validation of scar localization using histology confirmed the hallmark features of dense collagen deposition and overall tissue contraction (Fig. 4D).

**DISCUSSION**

Improved nondestructive and noninvasive imaging of the vocal fold mucosa would transform both preclinical research and clinical practice. MRI technology continues to advance at a rapid pace and is increasingly capable of meeting this imaging need. High- and ultrahigh-field instruments are commercially available; proof-of-concept and experimental animal studies conducted with such instruments can help guide the development of future clinical protocols. To this end, our goal was to further develop the applicability of MRI-based imaging of the vocal fold mucosa, using a preclinical rat model. We focused on naïve, acutely injured and chronically scarred vocal fold mucosa, as wound healing is relatively well conserved in humans and rats (Hirano et al., 2009; Tateya et al., 2005), scarring is primarily a subepithelial pathology (Gurtner et al., 2008; Martin, 1997), and because vocal fold scar can be challenging to assess using traditional imaging modalities (Dailey and Ford, 2006). Our data show that high- and ultrahigh-field MRI can resolve key anatomic features of the naïve rat larynx and its vocal fold mucosae, qualitative and quantitative elements of the acute injury phase, and the presence of chronic scar. This imaging was most successful with ex vivo specimens.

We resolved key anatomic features of the naïve and scarred vocal fold mucosae ex vivo using T1W imaging and Gd contrast enhancement. This paramagnetic, extracellular contrast agent distributes within the intravascular and interstitial spaces and differentially alters tissue contrast by shortening T1 relaxation time (Weinmann et al., 1984). At both 4.7 and 9.4 T, the naïve vocal fold mucosa—specifically its lamina propria—was hyperintense on T1W imaging compared with the adjacent intrinsic laryngeal muscles and cartilages. The vocal fold lamina propria is viscoelastic (Chan and Titze, 1999), anisotropic (with primary alignment of fibrous matrix proteins along its anterior–posterior plane) (Julias et al., 2013; Kelleher et al., 2014), and consists of an extracellular matrix rich in hydrophilic glycans such as hyaluronic acid (Gray et al., 1999). The tendency of these glycans to bind water makes the lamina propria ideal for MR signal enhancement (Sadeghi et al., 2003). Compared

Characterization of vocal fold scar

Mature vocal fold scar appears ~2 months following mucosal injury in the rat (Tateya et al., 2005; Welham et al., 2015). To evaluate our ability to resolve vocal fold scar tissue with MRI, we created unilateral vocal fold mucosal injuries and performed ex vivo imaging following this 2-month scar maturation period. The scarred mucosa appeared as a hypointense and volumetrically deficient region on T1W and T2W images (Fig. 4A); the greatest contrast with the hyperintense naïve mucosa was obtained with a T1W

at 3 days post-injury, and showed increased abundance at 5 and 7 days post-injury (Fig. 3E).

Fig. 2. Superparamagnetic iron oxide (SPIO) contrast enhancement of acute vocal fold injury. (A) T2- and T2*-weighted (T2W, T2*W) coronal images of the rat abdomen and neck, acquired in vivo at 4.7 T with and without intravenous SPIO contrast enhancement. Red asterisks indicate livers, red arrows indicate larynges. (B) T2W axial and coronal images of the rat larynx, 5 days following right-sided vocal fold mucosal injury. Images were acquired ex vivo at 4.7 T, with and without (pre-explant) intravenous SPIO contrast enhancement. Red arrows indicate hypointense mucosal lesions. (C) Pseudocolored volume renders of the vocal fold mucosal lesions shown in B. Lesions are red; thyroid (brown), cricoid (green) and arytenoid (cyan) cartilages are shown for anatomic orientation. (D) Effect of contrast enhancement on vocal fold mucosal lesion volume (mean±s.e.m.); n.s., no significant difference (P>0.01), calculated using a Student’s t-test. (E) H&E-, Prussian Blue- and CD68-stained vocal fold coronal sections, 5 days following mucosal injury. Black arrows indicate blood (red) and hemosiderin (brown) in the H&E-stained sections and ferric iron (blue) in the Prussian Blue-stained sections; white arrows indicate CD68+ cells (green) in the immunosections (nuclei are counterstained blue). Scale bars: 100 μm. Data represent n=5 animals per experimental condition in A–E, with the exception of the injury +SPIO images and render in panels B and C; these data represent n=2 animals in which contrast enhancement was associated with larger lesion volumes. R, right; L, left.
with the naive condition, vocal fold scar was characterized by a sharp decrease in lamina propria T1 signal intensity and overall mucosal volume, consistent with previous ex vivo ferret data collected at 11.7 T (Herrera et al., 2009). These MRI features corresponded to increased collagen abundance and tissue contraction; previous work with the rat model has also shown loss of hyaluronic acid (Tateya et al., 2005).

We evaluated the acute injury response using T2W imaging and observed peak hypointense lesion volume at day 5. T2W sequences are highly sensitive to the magnetic state of hemoglobin and its degradation products within an acute or subacute hemorrhage; in certain clinical scenarios such as brain hemorrhage, MRI is used to help stage the injury (Bradley, 1993). Classically, early acute hemorrhage is populated by a mixture of oxy- and deoxyhemoglobin-containing red blood cells. Both forms contain heme iron in its ferrous state; however, oxyhemoglobin is diamagnetic whereas deoxyhemoglobin – where the iron atoms contain four unpaired electrons – is paramagnetic. As the hemorrhage begins to mature, oxyhemoglobin is converted to deoxyhemoglobin, which in turn is oxidized to form methemoglobin (containing paramagnetic ferric heme iron atoms possessing five unpaired electrons). Finally, red blood cell lysis occurs, spilling methemoglobin into the extracellular space where it is further denatured into a range of iron-containing hemichromes and targeted for phagocytosis by macrophages. Macrophages accumulate iron and deposit it within the iron storage protein...
ferritin, which itself might degrade into hemosiderin. These physiologic changes typically yield maximum shortening of T2 relaxation time at the late-acute and subacute phases, when paramagnetic deoxyhemoglobin and methemoglobin predominate at the injury site (Bradley, 1993). Moreover, iron cores within ferritin and hemosiderin are superparamagnetic and possess T2 and T2* shortening properties similar to those of SPIO.

Our findings in rat vocal fold mucosa are consistent with these principles. Deoxyhemoglobin seemed to be present by day 1 post-injury, indicated by the initial hypointense lesion on T2W imaging, the acute hemorrhage on H&E staining, and the absence of ferric iron on Prussian Blue staining. Ferric iron was first detected at day 3, suggesting early oxidation of heme iron and, therefore, the presence of methemoglobin. Peak lesion volume at day 5 corresponded to a marked increase in ferric iron signal intensity, as well as the identification of CD68+ macrophages and intracellular hemosiderin—consistent with further oxidative denaturation and methemoglobin accumulation, in addition to hemichrome deposition and the beginning of phagocytosis. The decrease in lesion volume at day 7, combined with the persistence of ferric iron, suggests an ongoing progression from paramagnetic methemoglobin to hemichrome formation.

We were unable to clearly identify macrophage infiltration of the acute injury site using SPIO contrast enhancement at 5 days post-injury. Whereas certain SPIO-treated animals—such as the case shown in Fig. 2—exhibited increased lesion volumes, greater ferric iron abundance and greater CD68+ macrophage infiltration, there was no significant group difference in lesion volume between animals with and without SPIO. This finding might have resulted from the accumulation of multiple paramagnetic substances (endogenous deoxyhemoglobin and methemoglobin, as well as exogenous SPIO) at the lesion site at day 5, causing a saturation of T2 signal loss. It is well known that acute and subacute hemorrhage exhibit strong T2 hypointensity; hemorrhage can also contribute to regional magnetic non-uniformity and blooming artifact (Bradley, 1993). Therefore, despite peak cellular infiltration at day 5 (Ling et al., 2010a,b), the presence of SPIO might have had minimal impact on overall T2 hypointensity, compared with that of endogenous heme iron.

The rat vocal fold mucosa has dimensions of ∼1.0-1.4 mm along its anterior–posterior plane, ∼1.0 mm along its superior–inferior plane, and ∼0.2–0.5 mm along its medial–lateral plane, yielding a volume of less than 0.2 mm3 (Kurita et al., 1983; Ling et al., 2010a). Despite this relatively small volume, we were able to optimize MRI protocols for naïve, acutely injured and chronically scarred mucosae at 4.7 T by using an ex vivo tissue setup, immersion contrast enhancement, appropriate image weighting and extended acquisition times. These protocols yielded useful data at 41 μm³ voxel resolution, comparable with the 39 μm³/pixel resolution reported by Herrera et al. (2009) in (substantially larger) ex vivo ferret and dog larynges. Despite this progress ex vivo, practical implementation of MRI for the assessment of vocal fold substructures in experimental animals and human patients requires high-resolution in vivo scanning, which in turn requires higher field strengths, improved signal-to-noise ratios, and shorter acquisition times. Towards this end, we obtained equivalent spatial resolution and image quality with substantially less acquisition time when using a 9.4 T magnet. Even higher-resolution MRI is feasible in rodents in vivo at 16.4 and 21.1 T (Scheppin et al., 2010; Shahaj et al., 2012; Ullmann et al., 2013, 2014); in vivo human MRI has been reported with field strengths as high as 9.4 T (Pohmann et al., 2016). The use of a purpose-designed surface coil could provide further improvements in signal-to-noise ratio (McArdle et al., 1986). Additional considerations when translating this approach include the use of respiratory gating and (in the case of humans) behavioral strategies to reduce motion artifacts associated with breathing, swallowing and coughing (Ehman et al., 1984; Nygren et al., 2016).

In summary, our data demonstrate the potential of current preclinical MRI technology for the assessment of vocal fold subepithelial tissue changes in the rat model. Further progress, technology development and regulatory approvals might reduce the number of animals needed for preclinical studies as the vocal fold injury response and disease progression could be monitored using in vivo serial scans. Moreover, next generation high- and ultra-high-field MR instruments might one day assist clinicians and surgeons as they evaluate subepithelial changes to the vocal fold mucosa, consider differential diagnoses and engage in treatment decision making.

MATERIALS AND METHODS
Animals
Four-month-old Fisher 344 male rats (total n=47; Charles River, Wilmington, MA) were used for all experiments. All in vivo work was conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Act (7 U.S.C. et seq.). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison.

Vocal fold mucosal injury
Unilateral vocal fold mucosal injuries were created as previously reported (Ling et al., 2010a; Tateya et al., 2005). Briefly, rats underwent anesthesia induction using isoflurane (2.3%, mixed with 100% oxygen and delivered via induction chamber at 0.8-1.5 l/min) followed by maintenance using an intraperitoneal injection of ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (9 mg/kg). Atropine sulfate (0.05 mg/kg) was also injected intraperitoneally to reduce the secretion of saliva and sputum in the laryngeal lumen. The anesthetized rats were placed on an operating platform and a 1 mm diameter steel wire laryngoscope was inserted to facilitate vocal fold visualization. A 1.9 mm diameter, 25g rigid endoscope (Richard Wolf, Vernon Hills, IL) connected to an external light source and video monitor was used for surgical monitoring. The right vocal fold mucosa was stripped using a 25 gauge needle.

Animals in the acute injury experiment (n=5 per time point and SPIO condition) underwent in vivo MRI, followed by euthanasia and tissue harvest for ex vivo MRI, at 1, 3, 5 and 7 days post-injury; animals in the chronic scar experiment (n=5) underwent euthanasia and tissue harvest for ex vivo MRI at 2 months post-injury.

Magnetic resonance imaging
MRI was primarily performed in a 4.7 T instrument (Agilent Technologies, Santa Clara, CA) using a 210 mm bore magnet and standard volume coil. An additional high-field scan of the naïve larynx was performed in a 9.4 T instrument (Magnex Scientific, Yarnton, UK) using a 310 mm bore magnet and standard volume coil. VnmrJ software (Agilent Technologies) was used for instrument control and data acquisition.

In vivo scans were performed under isoflurane maintenance anesthesia (1%, mixed with 100% oxygen and delivered via nose cone at 1.0 l/min). Respiratory rate was monitored using a Safari model 1030 system (SA Instruments, Stony Brook, NY). For experimentally naïve rats, we began by acquiring T1W neck images without contrast enhancement. Next, to reduce longitudinal relaxation time and evaluate its effect on tissue contrast, we injected intravenous Gd contrast agent (0.5 mmol/kg MultiHance, Bracco Imaging, Princeton, NJ) and repeated the T1W acquisition sequence. For rats in the acute injury experiment, we acquired T2W and T2*W abdominal and neck images at each post-injury time point. To reduce T2 relaxation time and evaluate its effect on tissue contrast, we injected a subset of rats in the 5 day post-injury group with intravenous SPIO contrast agent [200 μmol Fe/kg Ferex...
rendering and volume measurements were performed using OsiriX 6.0 (MultiHU
cine) in 4% PFA for 10 days. For comparison, a small number of
content was acquired immediately following the in vivo scans and laryngeal
ease. All ex vivo samples were blotted to remove surface fluid and then suspended in liquid
fluorocarbon prior to scanning.
We used the following acquisition protocols at 4.7 T: (1) T1W gradient
echo, in vivo [15.5 ms repetition/echo time, 65° flip angle, 256×128×128
matrix, 70+35+35 mm field-of-view (FOV)]; (2) T2W gradient echo,
ex vivo (50/6.5 ms repetition/echo time, 65° flip angle, 512×256×256
matrix, 30+15+15 mm FOV); (3) T2W gradient echo (93/12 ms repetition/
24° flip angle, 45° flip angle, 256×128×128 matrix, 18×12×12 mm FOV); (4)
T2*W gradient echo (70/20 ms repetition/echo time, 20° flip angle,
128×128×128 matrix, 18×12×12 mm FOV). We used the following acquisition protocol at 9.4 T: T1W gradient echo, ex vivo (8.5/4 ms
repetition/echo time, 8° flip angle, 256×256×256 matrix, 12×20×20 mm
FOV).
Scan data were analyzed using ImageJ (Schneider et al., 2012). Volume
rendering and volume measurements were performed using OsinX 6.0
(Pixmeo, Bernex, Switzerland) and Amira 5.2 (Visage Imaging, Berlin,
Germany).

Histology and immunohistochemistry
All scanned larynges were processed for histology and/or
immunohistochemistry (IHC). Using whole laryngeal blocks, 6 μm frozen
serial sections were prepared in the coronal plane. Sections that included
the midmembranous vocal folds were retained for staining. Routine H&E
staining was used to evaluate cell and tissue morphology; routine Masson’s
Trichrome staining was used to evaluate collagen deposition. Prussian Blue
staining was used to detect ferric iron, as follows: sections were immersed in
a 1:1 cocktail of 20% hydrochloric acid and 10% potassium ferrocyanide for
20 min, rinsed in deionized water, counterstained with Nuclear Fast Red
solution (Newcomer Supply, Middleton, WI) for 5 min, dehydrated, and
coverslipped.
Sections intended for IHC were fixed using acetone for 10 min, washed
with phosphate-buffered saline (PBS), and blocked using 5% BSA (Sigma,
St Louis, MO) for 60 min. Next, sections were sequentially incubated with
mouse anti-rat CD68, clone ED1 (1:750; MCA341, AbD Serotec, Raleigh,
NC) for 90 min, followed by Alexa Fluor 488 goat anti-mouse IgG (1:800;
A11001, Life Technologies, Grand Island, NY) for 60 min, counterstained with DAPI (1:5000; Life Technologies) for 5 min, and coverslipped. Rat
spleen was used as a positive control. Negative control sections, stained
without the primary antibody, ensured each immunosignal was specific to
the intended antigen.
Images were captured using a microscope with both bright field and
fluorescent capabilities (E-600; Nikon, Melville, NY), equipped with a
digital microscopy camera (DP-71; Olympus, Center Valley, PA).

Statistics
Given the absence of existing MRI data characterizing lesion volumes
following acute vocal fold injury, we powered this experiment using
histologic measures of rat vocal fold mucosal cross-sectional area at our
post-injury time points of interest (Ling et al., 2010a). Based on these data,
we estimated that n=5 animals per time point would allow detection of a
>1 s.d. shift in mean lesion volume with 80% power. Animals were not
randomized. All image analysis procedures were performed on blinded
samples.
No data points were removed prior to statistical analysis. Data were
evaluated for normality and equality of variance using visual inspection of
raw data plots and Levene’s test. The data did not meet the normality
assumption and were therefore rank-transformed prior to additional testing.
Lesion volume data were analyzed using a Student’s t-test for the
comparison of injury and injury+SPIO conditions at 5 days post-injury
(Fig. 2D), and one-way analysis of variance (ANOVA) for assessment of the
acute post-injury time course (Fig. 3C). In the ANOVA model, as the F-test
showed a significant difference across time points, Fisher’s protected least
significant difference method was used for planned pairwise comparisons.
A type I error rate of 0.01 was used for all statistical testing; all P-values were
two-sided.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
N.V.W., I.J.R. and A.O.K. conceived the study and designed the experiments. N.V.W.
obtained funding. A.O.K., Y.K. and D.L.Y. conducted the in vivo experiments and
performed the ex vivo tissue work. I.J.R. and J.Z. collected and analyzed the MRI data.
A.O.K. and D.L.Y. performed histology and immunohistochemistry. A.O.K. wrote the
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