Tissue-resident memory CD8 T (TRM) cells are a unique immune memory subset that develops and remains in peripheral tissues at the site of infection, providing future host resistance upon reexposure to that pathogen. In the pulmonary system, TRM are identified through S1P antagonist CD69 and expression of integrins CD103/β7 and CD49a/CD29(β1). Contrary to the established role of CD69 on CD8 T cells, the functions of CD103 and CD49a on this population are not well defined. This study examines the expression patterns and functions of CD103 and CD49a with a specific focus on their impact on T cell motility during influenza virus infection. We show that the TRM cell surface phenotype develops by 2 wk postinfection, with the majority of the population expressing CD49a and a subset that is also positive for CD103. Despite a previously established role in retaining TRM in peripheral tissues, CD49a facilitates locomotion of virus-specific CD8 T cells, both in vitro and in vivo. These results demonstrate that CD49a may contribute to local surveillance mechanisms of the TRM population.

Significance

Current influenza vaccination strategies require annual immunizations, with fairly low efficacy rates. One technique to improve protection against a greater breadth of influenza viruses is to elicit broadly cross-reactive cell-mediated immunity and generate a local population of cytotoxic T cells to respond to conserved regions of circulating viruses. However, this approach requires improved understanding of how these cells migrate within and attach to the tissue in order to persist and offer long-term immunity. This study investigates how receptors on the T cell surface impact the cell’s ability to interact with the tissue and provide evidence of which of these receptors are essential for protection. Furthermore, these studies reveal functional in vivo mechanisms of cellular markers used to characterize TRM.

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essential for optimal TRM maintenance but do not delve into the direct interactions that lead to these functional ramifications. Therefore, we investigated how these integrins regulate interactions with the tissue through adherence and motility with the ultimate aim of determining whether these integrins equally contribute to host protection.

Results

Pulmonary CD8 T Cells Express CD49a and CD103 Early after Influenza Virus Clearance. TRM cells are classically identified through expression of CD69 and integrins CD103 and CD49a. CD69 limits egress of TRM cells from tissues by acting as an antagonist to S1P and reduces responsiveness to S1P1 gradients that direct the cells toward the draining lymphatics (25, 26). The functions of the surface integrins CD103 and CD49a on T cells are less well appreciated. Additionally, the temporal development of this surface phenotype is not well characterized. Bona fide TRM cells can be found in the airways, lung tissue, and trachea at 3 mo postinfection (2), and these cells express CD49a and/or CD103. We asked whether this phenotype arose early after virus clearance by comparing the integrin phenotypes at 2 wk postinfection, with TRM cells present at 3 mo. CD8 T cells were examined in two different models of influenza virus infection. In the first, C57BL/6 mice were infected intranasally with H3N2 influenza A virus HKx31. In the second model, which is used for all imaging-based experiments described in this study, GFP OT-I cells were adoptively transferred prior to infection with an HKx31 variant expressing the Ova1 SIINFEKL peptide in the neuraminidase stalk (27). To clearly separate cells in the tissue from cells circulating or intimately associated with the vasculature, antibody was introduced intravascularly prior to harvesting cells from the airways, tracheal tissue, or lung tissue (28, 29). Close to 100% of virus-specific CD8 T cells in the airways were negative for intravascular labeling, indicating that almost all of the cells are within the tissue parenchyma (SI Appendix, Figs. S1 and S2). At 3 mo after HKx31 infection, greater than 80% of NP/PA tetramer (Tetramer+) or by gating on OT-I CD8 T cells (OT-I) at 3 mo or 14 d postinfection. Cells were examined in the airways through bronchoalveolar lavage (BAL), lung tissue (B), and trachea (C). Results shown are mean ± SD from one representative of three independent experiments.

CD49a and CD103 Differentially Affect T Cell Behavior In Vitro. CD49a and CD103 have been shown to bind and interact with collagen and E-cadherin, respectively (30–34). However, in T cells, the understanding of how these interactions regulate motility is not fully established. Using a two-dimensional (2D) in vitro system, we first asked the question, Do CD49a and CD103 facilitate migration? On day 14 postinfection, virus-specific CD8 T cells identified through expression of GFP were imaged on mouse collagen IV (four) or E-cadherin. GFP OT-I T cells on collagen IV displayed robust motility, with median speeds of 4.48 μm/min (IQR of 3.85), displacement rates of 0.79 μm/min (IQR of 2.29), and directionality as measured by straightness of 0.20 (IQR of 0.33) (Fig. 2A–D). The addition of CD49a blocking antibody resulted in reduced speeds, displacement, and straightness, suggesting that the migration was mediated through the CD49a-collagen IV interaction (Fig. 2E–G and Movie S1). To verify that the observed effect was not indicative of a deterioration of the health of the cells, an antibody against CD103 was added to cells migrating
CD49a and CD103 differentially affect T cell behavior in vitro. OT-I T cells were extracted and negatively enriched from influenza-infected lung at day 14 postinfection. Cell motility was quantified on mouse collagen IV or E-cadherin before and after addition of blocking antibody. Speed (track length/time) (A), displacement rate (displacement/time) (B), and straightness (total track length/displacement) (C) were calculated. Representative tracks from one experiment are shown, with color indicating average track speed (D–F and I–K). Each wild-type condition includes data from six independent experiments and each treatment condition includes data from three independent experiments. Representative TIRF images on collagen and E-cadherin with arrows showing examples of cellular processes (G and I). Number of processes, shown as median with range for six individual cells on each surface (H and M). Data in A–C are from six control mice and three for each experimental condition. The red bar indicates the median. Significance was first established within each independent experiment and also with compiled data. *P < 0.05.

We have shown that CD8 T cells can express CD49a and/or CD103 by flow cytometry and that these integrins provide distinct motility functions when examined in vitro. However, in both sets of experiments, cells were extracted from the tissues, which may not be representative of the entire population and does not indicate cellular location of the integrins (35). To identify the cellular localization of CD49a and CD103, tracheal tissue whole mounts from mice at days 14, 21, and 42 and 3 mo postinfection were stained and examined by microscopy. By day 14 postinfection, CD49a could be identified in proximity to collagen as measured by second-harmonic generation, directly underlying the epithelium (Fig. 3A). Within the epithelial layer, predominantly CD103-expressing cells were identified (Fig. 3A). At days 21 and 42 postinfection, it was clear that higher expression of these integrins on the cell surface was localized to specific foci, and it was more common to see expression of a single integrin type in a given region (Fig. 3B and C). This suggested that some cells may be utilizing only one interaction or that a cell could be interacting with multiple substrates at distinct cellular regions simultaneously. By 3 mo postinfection, we observed a similar decrease in cells, as seen by flow cytometry, and identifying cells at this time point became more challenging. However, we could find small numbers of dual-expressing cells within the tissue as well as CD103+ cells embedded within the epithelium (Fig. 3D). Of the cells identified at this time point within the epithelium, we could observe CD103 expression not only on the cell bodies but also on cellular processes in between epithelial cells (Fig. 3D). We were similarly able to identify CD8+ cells in human trachea obtained from the Lung Development Molecular Atlas Program (LungMAP) consortium, the LungMAP Biorepository, and the LungMAP Data Coordinating Center. We observed CD103-expressing cells...
closer to the tip of the airway epithelial cells (upward-facing arrows) and dual-expressing CD8+ cells closer to the basement membrane (Fig. 3E), suggesting that integrins on human CD8 T cells may employ similar interactions.

CD49a Promotes Motility and CD103 Limits Speed In Vivo. One main goal of this study was to evaluate the role(s) of these integrins in cell motility in vivo. To achieve this, we utilized an intravital tracheal imaging system, previously developed in the laboratory (36). Transgenic OT-I T cells from wild-type (WT) or integrin-deficient mice were transferred to wild-type hosts and imaged at 2 wk postinfection to examine CD8 T cell movement over time. In comparison to the simplistic 2D collagen system, wild-type cells observed in the trachea displayed fairly low speeds (median of 1.95 μm/min with an IQR of 1.82), without extensive displacement (median of 0.33 μm/min with an IQR of 0.41) (Fig. 4A–D and Movie S5). This is likely due to the combination of signals the cells are receiving through multiple interactions and a consequence of being in a more confined environment. However, eliminating CD49a in GFP OT-I cells resulted in further limited motility, consistent with the concept that CD49a is facilitating locomotion on collagen IV (Fig. 4A–C and E and Movie S6). Conversely, the absence of CD103 resulted in increased speeds compared with wild-type cells (Fig. 4A–C and F and Movie S7). The number of cells at this time point, however, was reduced, so we employed a second approach to examine the contribution of CD103 in this system. Wild-type OT-I cells were transferred into naïve wild-type hosts, and starting on day 7 postinfection, mice were given a CD103-blocking antibody or isotype antibody every other day until imaging. To ensure the
antibody was reaching the cells, separate mice were examined for the presence of the antibody bound to virus-specific CD8 T cells, using an anti-rat IgG antibody. Cells in the trachea, lung, and airways of anti-CD103 treated mice were labeled with the anti-rat IgG, while isotype control mice showed no binding of the secondary antibody (SI Appendix, Fig. S5A). The absence of staining with the CD103 antibody prior to flow cytometric analysis suggested that not only was the in vivo antibody already bound but it was likely saturating. Blocking CD103 using this approach resulted in numbers of cells comparable to wild type on day 14 postinfection. However, the motility was markedly similar to that observed using the knockout cells. OT-I CD8 T cells displayed increased speeds in anti-CD103-treated mice compared with controls (Fig. 4 G–J and Movie S8). This effect was moderate, likely reflecting the fact that only about a third of CD8 T cells express CD103 and, therefore, two-thirds of the cells are unaffected by the antibody. The data support the conclusion that CD103 is not a mediator of motility, suggesting a role in localization and/or cell–tissue interactions.

**CD49a and CD103 in T<sub>RM</sub> Formation and Function.** Both integrins have been implicated in the formation of T<sub>RM</sub> in the tissue. To determine whether the absence of one or the other integrin affects the overall integrin phenotype early after viral clearance, cells were examined using flow cytometry after transfer of either WT or integrin-deficient cells on day 14 postinfection. In the absence of CD49a, no changes in the frequency or number of CD103 positive cells were observed (Fig. 5A and B). Likewise, cells deficient in CD103 expressed similar frequencies of CD49a at this same time point (Fig. 5C and D). The total number of CD103-deficient CD8 T cells expressing CD49a was slightly, but not significantly, decreased (Fig. 5D). However, this was not unexpected based on the in vivo data. Overall, our data suggest that the level of each integrin is regulated independently of the other. However, there could still be functional compensatory mechanisms.

Therefore, we next sought to determine whether CD49a and CD103 could equally contribute to host defense. Our laboratory previously showed a requirement for CD49a to protect a host from heterosubtypic influenza virus challenge at 3 mo postinfection (2). However, the contribution of CD103 in secondary protection has not been established. To examine this, WT and CD49a-deficient mice were infected with the H3N2 virus HKx31 to generate a pool of memory cells. Three days and 1 d prior to reinfection with H1N1 virus PR8, both genotypes of mice were given either CD103-blocking antibody or phosphate-buffered saline (PBS) control. Again, to ensure localization of the antibody, separate mice were examined for binding of a secondary
antibody to show the presence of anti-CD103 (SI Appendix, Fig. S5B). CD49a-deficient mice, regardless of treatment, succumbed to the challenge by day 8, only 1 d later than nonimmune mice with no prior infection (Fig. 6A and B). WT mice given CD103-blocking antibody were protected equally as well as mice given control treatment, and the majority of mice survived the challenge (Fig. 6A and B). These data show a clear requirement for CD49a but further suggest that the CD103–E-cadherin interaction is not necessary at late time points for TRM-mediated host protection.

**Discussion**

Integrins CD49a and CD103 appear on the surface of CD8 T cells in the airway, trachea, and lung tissue early after clearance of influenza virus, by day 14. While these integrins are viewed as markers of TRM cells, they actually offer CD8 T cells in the airways, trachea, and lung tissue early after clearance of influenza virus, by day 14. While these integrins are viewed as markers of TRM cells, they actually offer CD8 T cells a way to interact with collagen IV in the lamina densa underlying the epithelium, collagen I in the interstitium, and the epithelial cells directly through E-cadherin binding. The expression of CD49a and CD103 early after viral clearance suggests they are playing a critical role contributing to the development and maintenance of TRM. Previous work has established that active TGF-β, which is present during disease resolution, is essential for expression of CD103 and CD49a (37).

In the gut, this has been highlighted by eliminating TGF-β signaling to T cells, which results in continued recruitment of effector cells but no demonstrable population of tissue memory (37). Additionally, Nur77 signaling was recently linked to direct regulation of CD49a transcription in endothelial cells, potentially implicating a role for in situ TCR stimulation in modifying surface expression in this system (38, 39). Integrins can also be regulated through outside-in signaling, suggesting that direct interactions with collagen or E-cadherin on cells poised within or in proximity to the airway may enhance surface expression of CD49a or CD103, respectively (40).

Distinct subsets of CD8 T cells in the airway, lung tissue, and trachea can be identified through expression of CD49a and/or CD103. Early characterization of TRM cells focused exclusively on CD103 and CD69 as identifiers of the population; however, our laboratory has shown that CD49a is critical for long-term maintenance of the population in the lungs, in part through survival signals received via interactions with collagen IV (2, 16). CD103 appears to regulate early recruitment and persistence but is less critical after resolution of infection for maintenance of the memory pool (21, 22). We sought to examine the outcomes of these integrins interacting with their ligands both in vitro and in vivo to reveal potential mechanisms regulating the TRM population and ultimately host protection.

In vitro imaging of virus-specific CD8 T cells on collagen IV and E-cadherin suggested that only CD49a mediated locomotion in the context examined. CD8 T cells migrated readily on collagen IV in a CD49a-dependent manner. While previous reports examining other cell types such as fibroblasts and endothelial cells suggested that CD49a’s function was primarily to adhere to collagen, the differences observed could be attributed to distinct mechanisms mediating adhesion between cell types (38, 41, 42). Inhibition or knockdown of CD49a in tumor cells resulted in decreased migration compared with their CD49a-sufficient counterparts (43, 44). Similarly, human CD8 T cells in vivo were unable to migrate through a collagen IV–coated transwell when CD49a was blocked (18). More extensive examination of whether these CD8 T cells on collagen require active reengagement of ligand to survive long term is warranted, with the knowledge that the CD49a–collagen IV interactions are important for cell persistence. Additionally, it would be of interest to determine whether these outcomes are specific to collagen IV or if these phenomena can be more generally applied to other collagens, such as collagen I, which is distributed more broadly throughout the tissue. Alternatively, T cells on E-cadherin displayed attachment to the surface but exhibited limited migration. This could suggest a fundamentally different role in regulation of cell behavior, and this is consistent with both lymphoid and nonlymphoid cells examined in other organs such as the gut (33, 34). This interaction may mediate local retention of cells at the peripheral site and provide continued surveillance. While we observed an increase of cellular processes on the cells on E-cadherin, further investigation will be necessary to determine if this is due solely to the interaction between CD103 and E-cadherin or if this observed morphology is downstream of inside-out cytokine signals or requires the contribution of other adhesion molecules (20, 45). Salivary gland TRM and some dendritic cells have been shown to express E-cadherin (46, 47), which could participate in binding through homotypic interactions.

Within the tissue, CD49a and CD103 are not uniformly distributed on the cell surface. In fact, we can find expression of
each integrin in regions of the integrin ligand. CD103 coincided with E-cadherin expression within the epithelium, consistent with the capacity to interact with epithelial cells. CD49a could be identified in close proximity to collagen directly underlying epithelial cells, suggesting it may use this surface to explore other regions of the epithelium and receive prosurvival signals. To determine whether these integrins played similar roles in vivo to what was observed in vitro, we used a combination of genetically deficient T cells and blocking antibodies to examine the motility of virus-specific CD8 T cells in the trachea of 2 wk infected mice. Consistent with cell motility observed in the 2D setting, the absence of CD49a in vivo resulted in further arrest of the cells, indicating a role in cell locomotion. This observation conflicted with our initial interpretation that CD49a was important for retention by physically adhering the cells to the matrix. Instead, these studies reveal that CD49a is critical for cell motility, a feature that was not previously associated with retention. In contrast, knocking out or blocking CD103 resulted in increased speeds of the cells examined. While in vivo migration of T cells lacking CD49a has not been extensively examined, transplanted human skin in a mouse model of psoriasis showed that CD49a blockade limited the ability of T cells to position within the epidermal layer and sequestered these cells to the dermis, signifying an important role for CD49a in motility and/or positioning (18). Alternatively, imaging of CD103-deficient CD8 T cells in the skin during a mouse model of herpes simplex virus infection similarly displayed increased speeds, suggesting that CD103 restrains the cells’ motility, possibly by affecting tissue localization or tissue interactions, which remain to be investigated (20).

For years, the CD8 T cell field has relied on using integrins CD103 and CD49a to identify the TRM subset; however, our understanding of the functions of these receptors in regulating interactions with the tissue and T cell motility is incomplete and underappreciated. In this paper we examined the roles of CD103 and CD49a to identify the TRM subset; however, our understanding of the functions of these receptors in regulating interactions with the tissue and T cell motility is incomplete and underappreciated. In this paper we examined the roles of CD103 and CD49a in the CD8 T cell subset present after clearance of viral infection. The absence or blocking of CD49a in this system showed an unexpectedly strong effect, indicating that other integrins such as CD51 which confer motility in CD4 T cells are not sufficient to compensate in this population (48). Alternatively, imaging of CD103-deficient CD8 T cells in the skin during a mouse model of herpes simplex virus infection similarly displayed increased speeds, suggesting that CD103 restrains the cells’ motility, possibly by affecting tissue localization or tissue interactions, which remain to be investigated (20).

Materials and Methods

Mice. All mice were housed in university-approved microisolator cages, within a pathogen-free facility. C57BL/6J mice (Jackson Laboratories) used for experiments were infected with 8 to 10 wk of age. OT-1 GFP transgenic mice were maintained in house at the University of Rochester (36). This line of mice was crossed with VLA-1 knockout (KO) mice (2) or CD103 KO mice [B6.129S2(C)− ; mice. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals as defined by the NIH (50). Animal protocols were approved and reviewed by the Institutional Animal Care and Use Committee (IACUC) at the University of Rochester. All procedures were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals as defined by the NIH (50). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Rochester.

Treatment and Cell Transfer. On day −1, 1 × 106 splenocytes from WT or integrin KO GFP OT-1 cells were injected IV into naive C57BL/6J hosts. WT and integrin KO mice were checked by flow cytometry to ensure that comparable numbers of CD8 T cells were being transferred. For CD103 blocking, mice were given 150 μg anti-CD103 antibody (clone M290) or isotype control antibody (clone 2A3) intraperitonally in a 200 μL volume. Mice were given injections on days 7, 9, 11, and 13. For blocking prior to secondary challenge, mice were given 150 μg anti-CD103 or PBS on days −3 and −1 and 150 μg intranasally on day −2. For cellular preparations, 0.2 μg CD8β-FITC, CD45-PE, or CD45-Brilliant Violet 786 was given IV in 100 μL PBS 3 min prior to organ harvest.

Viruses and Infections. Mice were infected as previously described (36, 51). In brief, H3N2 A/Hong Kong/X31 (HKx31) influenza virus, HKx31-OVA1 expressing the ovalbumin (OVA257-264 SIINFEKL) peptide in the neuraminidase viral protein, and H1N1 A/Porto Ricos/ (PR8) were grown and stored in eggs as described in embryonated chicken eggs. Infection of CD103-deficient intranasally with 103 50% egg infectious dose (EID50) of HKx31, 3 × 10 5 EID50 HKx31-OVA1, or 10 4 EID50 PR8 in 30 μL of PBS. Infected mice were monitored daily for weight loss and other signs of morbidity.

Cellular Preparations. Bronchoalveolar lavage (BAL), lung, and tracheal samples were prepared as previously described (51). Briefly, BAL was collected by flushing the lungs with 1× PBS, and samples were lysed with ACK lysis buffer (ammonium–chloride–potassium). Lungs and trachea were dissected by the gentleMACS (Miltenyi Biotec) and were incubated in Collagenase II (Worthington) at 37 °C for 30 min, with gentle agitation. Cell suspensions were filtered and separated by a 75.40 Percoll (GE Healthcare) discontinuous gradient. Cell suspensions were counted on a hemocytometer, with trypan blue exclusion.

Flow Cytometry. Staining was achieved as previously described (51). Briefly, single-cell suspensions were stained in PBS with purified CD16/32 (clone 2.4G2); fixable live/dead indicator (Invitrogen); some combination of the antibodies TCR, CD8, CD45, CD49a, CD103, and CD44 (Biolegend or BD Biosciences); and influenza tetramers (NP and PA) (NIH Tetramer Core Facility). Samples were run on an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star).

In Vitro Imaging. Cell migration chambers (Millicell EZ slice eight-wall glass, Millipore, or Delta T dish, Biotech) were coated with 10 μg/cm2 mouse collagen IV (Corning) in 0.05 M HCl for 1 h at room temperature or 2.5 μg/cm2 mouse E-cadherin (R&D Systems) overnight at 4 °C in PBS. Prior to imaging, the plate was washed three times in PBS. For in vitro migration imaging, GFP expression was determined using confocal microscopy. Cells were imaged as described (51). For imaging with the time lapse setup, samples were kept at 37 °C with 5% CO2 with a stage top incubation system (Oko-Lab). For automated process detection in MATLAB, single Z plane TIRF microscope images of cells over time were preprocessed with intensity thresholding prior to generating a binarized version. (https://github.com/tophamlab/20projections/blob/master/projections.m). The Image Open software was applied to identify the inside perimeter of the cell body. This created a new image that represented only the cell body, and subtraction of this image from the original generated potential cellular processes. Object detection and local thresholding were used to distinguish cellular processes from noise. Finally, each cellular process was labeled and counted, and the number of processes for each cell at a
Intravital Multiphoton Imaging. Excised mouse trachea was incubated in PBS, serum, anti-CD16/32, and indicated antibodies for 4 h at room temperature. The following antibodies were used in different combinations at the indicated dilutions: CD103–Alexa Fluor 594 (Biolegend), 1:50; CD49a-Alexa Fluor 647 (BD Biosciences), 1:25; and E-cadherin–APC (Biolegend) 1:50. Images were acquired on an Olympus FV1000 (Center for Advanced Light Microscopy and Nanoscopy Shared Resource Laboratory) using an Olympus Plan Apo 60×1.43 NA objective. Fresh, transplant donor–quality human tracheal tissue was obtained from the Human Tissue Core of LungMAP at the University of Rochester. The University of Rochester Institutional Review Board approved and oversees this study (approval no. RSBR0047606). Human trachea was stained for 72 h in PBS serum and the following Biolegend antibodies: hCD103–Alexa Fluor 594, 1:40; hCD8–Alexa Fluor 488, 1:40; hCD49a–Alexa Fluor 647, 1:40; and E-cadherin–APC, 1:40. Human tissue was washed in PBS and fixed in 4% paraformaldehyde for 2 h prior to mounting. Mouse tissue was washed in PBS and mounted unfixed. All whole-mount tissue sections were mounted using Fluoromount-G (SouthernBiotech).

Intravital Multiphoton Imaging. Intravital tracheal imaging was performed as described in Lambert Emo et al. (36). Briefly, mice were anesthetized with 65 mg/kg pentobarbital. Hair was removed from one hind leg, exposing skin for MouseOX Plus sensor (Starr Life Sciences) monitoring. The hair was removed from the thoracic area with scissors, and the mouse was placed on a heated stage. Once the surgical plane of anesthesia was determined by both lack of pedal and palpebral reflex, the coat was opened between the chin and the top of the rib cage. The submandibular salivary glands were separated to reveal the muscles covering the trachea, and these muscles were separated to expose the trachea. A small flexible plastic support was placed under the trachea to separate it from the surrounding muscle, tissue, and coat. A small incision was made between cartilage rings below the larynx. An 18-gauge steel cannula was inserted into the opening in the trachea just below the sternum. Mice were ventilated through the cannula using the Harvard Inspira ASV ventilator with 100% oxygen and 0.5% isoflurane. Mice were ventilated through the cannula at rates maintained between 250 and 600 beats per minute. The rectal body temperature was monitored throughout the imaging session. Oxygenation levels were maintained at >95%, and heart rate was maintained between 200 and 600 beats per minute. The intrathoracic body temperature was monitored and maintained using a small-animal feedback-regulated heating pad. After the animal was stable and reversionifican of the lack of both pedal and palpebral reflex, pancuronium bromide (0.4 mg/kg) was administered intramuscularly. Agarose (0.05%) was added to the exposed trachea, and plastic wrap was used to create a water basin for the mouse during the imaging process. All images were collected by an Olympus FVMPE-RS system (Olympus) and were analyzed and visualized using Imaris (Bitplane) software.
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