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Original Research

Allograft Inflammatory Factor 1 as an Immunohistochemical Marker for Macrophages in Multiple Tissues and Laboratory Animal Species

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Allograft inflammatory factor 1 (AIF1) is a commonly used marker for microglia in the brains of humans and some animal models but has had limited applications elsewhere. We sought to determine whether AIF1 can be used as a macrophage marker across common laboratory animal species and tissues. We studied tissues (that is, spleen, liver, and lung) with defined macrophage populations by using an AIF1 immunostaining technique previously validated in human tissue. Tissues were collected from various mouse strains (*n* = 20), rat strains (*n* = 15), pigs (*n* = 4), ferrets (*n* = 4), and humans (*n* = 4, lung only). All samples of liver had scattered immunostaining in interstitial cells, consistent with resident tissue macrophages (Kupffer cells). Spleen samples had cellular immunostaining of macrophages in both the red and white pulp compartments, but the red pulp had more immunostained cellular aggregates and, in some species, increased immunostaining intensity compared with white pulp. In lung, alveolar macrophages had weak to moderate staining, whereas interstitial and perivascular macrophages demonstrated moderate to robust staining. Incidental lesions and tissue changes were detected in some sections, including a tumor, inducible bronchus-associated lymphoid tissue, and inflammatory lesions that demonstrated AIF1 immunostaining of macrophages. Finally, we compared AIF1 immunostaining of alveolar macrophages between a hypertensive rat model (SHR strain) and a normotensive model (WKY strain). SHR lungs had altered intensity and distribution of immunostaining in activated macrophages compared with macrophages of WKY lungs. Overall, AIF1 immunostaining demonstrated reproducible macrophage staining across multiple species and tissue types. Given the increasing breadth of model species used to study human disease, the use of cross-species markers and techniques can reduce some of the inherent variability within translational research.

Abbreviation: AIF1, allograft inflammatory factor 1

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Animal models are commonly studied in the preclinical setting to investigate human diseases. A goal of translational research is the efficient transfer of relevant findings from the animal model to the human condition.[5] However, differences in markers or techniques between animal models or between animal and human studies can introduce new sources of error and limit the reproducibility of a study's findings. If the tools and techniques applied in preclinical studies were similar to those of clinical studies, the results might be more translatable, thus maximizing the immediate relevance and clinical significance of the preclinical work.[42,43]

In pathology studies, labeling techniques are used to identify specific cellular and tissue markers.[11] In animal models, it is not uncommon to use labeling techniques (for example, immunohistochemistry) that are model-specific rather than directly translatable. However, optimized and validated immunohistochemical techniques that are applicable across species might be advantageous not only for potential significance and translation but also for cost efficiency in the laboratory.[11,30,36]

Inflammation is a common and critical component of many diseases[7,31] and is morphologically characterized by the presence of immune cells at histopathology. Macrophages are important immune cells that are quiescent residents in tissues and become activated through different pathways to mediate host defense and inflammation.[32] A wide variety of markers have been used to identify macrophages across species, but these often do not overlap in function or specificity between species. For example, F4/80 has been used as a marker for murine macrophages for decades, but humans lack this marker, and the homolog of this molecule—EGF-like module-containing mucin-like hormone receptor 1—is restricted to eosinophils, not macrophages.[14,16] Identification of immunostaining techniques that have both a common marker or labeling technique (or both) would be useful for translational investigations.[31] In the current study, we

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evaluated whether allograft inflammatory factor 1 (AIF1) was an effective marker for detecting macrophages in experimental tissues from common laboratory animal species.

Materials and Methods

Tissues. All tissues (Table 1) were collected from animals euthanized under approval of the University of Iowa IACUC. All IACUC-approved animal protocols were performed in accordance with the Animal Welfare Act,[3] Animal Welfare Regulations,[4] the *Guide for the Care and Use of Laboratory Animals*,[19] and the *AVMA Guidelines for the Euthanasia of Animals* (2013 edition).[2] Pig tissues were acquired from archival formalin-fixed, paraffin-embedded blocks from IACUC-approved studies. All other tissues (mouse, rat, and ferret) were harvested immediately after euthanasia by the IACUC-approved investigators and fixed in 10% neutral buffered formalin. In an effort to apply 3Rs principles (replacement, reduction, and refinement), these supplemental tissue samples were collected from healthy animals that were euthanized for other studies and included both naïve and experimental animals. Rodents were housed in a nonbarrier facility with quarterly health surveillance using dirty-bedding sentinels. Sentinels were consistently negative for adventitious agents. This strategy allowed for animal tissues to be shared (with investigator permission) and used in multiple studies, thereby reducing animal use; this process provided a broad variety and number of background strains for rodent tissues (Table 1). Because of the diversity of sources (age, sex, and so forth), we focused our evaluation to the species level.

The collection and use of human lung tissue was approved by the Institutional Review Board (University of Iowa); these de-identified samples were collected from archival formalin-fixed, paraffin-embedded tissue blocks. From these samples, regions of inflamed and noninflamed lung were identified. As an additional experiment, we evaluated AIF1 immunostaining of alveolar macrophages in a model of early hypertension; differences in the immune response of hypertensive compared with normotensive rats have been reported.[13] We collected lung tissues from male Wistar Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHR; age, 2.5 mo), because SHR develop spontaneous systemic hypertension, whereas WKY rats do not.[17]

Immunohistochemistry. Immunohistochemistry for AIF1 (previously known as ionized calcium-binding adapter molecule 1, IBA1; or IFNγ-responsive transcript 1) was performed as previously validated for the detection of microglia in the brains of humans and pigs.[30] Briefly, formalin-fixed, paraffin-embedded tissues were sectioned (thickness, approximately 4 µm), placed on glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA), baked (60 °C, 60 min; Isotemp Oven, Fisher Scientific), and hydrated through a series of xylene and alcohol baths. Heat-induced epitope retrieval was performed in a citrate buffer bath at pH 6.0 (125 °C, 5 min; model DC2002, Decloaking Chamber, BioCare Medical, Concord, CA). Slides were incubated in primary rabbit antiAIF1 polyclonal antibody (dilution, 1:1000; catalog no. 019-19741, AntiAIF1/IBA1, Wako Chemicals, Richmond, VA) for 1 h room temperature. After washing, the secondary reagents (Rabbit Envision HRP System, Dako, Carpinteria, CA) were applied according to instructions. Chromogen (DAB Plus, Dako) was applied to tissues for 5 min (room temperature), followed by DAB enhancer (Dako) for 3 min, and then counterstain (Surgipath Hematoxylin, Leica Microsystems, Wetzlar, Germany) for 1 min. Finally tissues were dehydrated through a series of alcohol and xylene baths and coverslipped.

For all samples, tissue morphology was examined by 2 reviewers (KMD and DKM), one of whom is a veterinary comparative pathologist (DKM). For semiquantitative and quantitative evaluation of tissues, we followed key principles for scoring of tissues, such as evaluating immune cells.[31] All scores were determined by the same reviewer, to decrease interobserver variation, and masking was performed by using the postexamination method.[12]

Staining (that is, brown coloration) intensity of AIF1 in the spleen was evaluated by using a semiquantitative scoring system: 0, absence of brown staining; 1, mild brown staining that did not obstruct viewing of the blue cytoplasmic counterstain; 2, moderate, distinct brown staining that partially obstructed viewing of blue cytoplasmic counterstain; and 3, strong, robust dark brown staining that completely obstructed viewing of the blue cytoplasmic counterstain. Splenic red and white pulp were graded separately for AIF1 staining intensity and compared. This staining intensity assessment was adapted from previously described approaches and methods.[1,28] For the evaluation of the hypertensive model, lung data for WKY (n = 3) and SHR (n = 3) rats were digitally collected from 2 random fields (magnification, 100×) for each animal. A masked observer ranked these images (n = 12) from least (1) to most (12) activated according to macrophage activation based on morphology. Specifically, activated alveolar macrophages were examined for larger nuclei and cytoplasm and for cytoplasmic foamy change as compared with quiescent cells; ranked scores (n = 2 per animal) were averaged for each animal for subsequent statistical analysis. Quantitative evaluation of macrophage diameter was made for each digital image, and these results were pooled for each animal. Scoring data were analyzed (Prism, GraphPad Software, San Diego, CA) by using a Wilcoxon matched-pairs signed rank test for splenic samples and unpaired t-tests for lung samples. Results were considered significant at a P value less than 0.05.

Results

Liver samples across species showed multifocal, moderate to robust, AIF1 immunostaining in scattered interstitial cells consistent with resident hepatic macrophages (Kupffer cells; Figure 1 A). Hepatocytes were negative for AIF1 immunostaining across species. Lung specimens had moderate to robust AIF1 immunostaining in scattered interstitial macrophages, but AIF1 immunostaining was often weak to moderate in most alveolar macrophages (Figure 1 B). In spleen (Figure 1 C), moderate to robust AIF1 cellular immunostaining was detected in the red pulp, with infrequent immunostaining in the white pulp. This relative distribution between red and white pulp macrophages is expected.[49] Comparison of immunostaining intensity showed that AIF1 immunostaining was significantly ($P < 0.05$) more robust in red pulp compared with white pulp macrophages of mice and rats, but the difference did not reach statistical significance in ferrets or pigs (Figure 1 D).

A few tissue changes and lesions were discovered incidentally during the study and provided an opportunity to observe patterns of AIF1 immunostaining in these situations. A papillary adenoma in the lung of a mouse had scattered robust AIF1 immunostaining in macrophages within the tumor (Figure 2 A). A rat lung contained a focal region of inflammation with the margination of $AIF1^+$ cells along the vessel wall (Figure 2 B). In another rat lung, a lymphoid aggregate consistent with inducible bronchus-associated lymphoid tissue was detected near a branching airway.[33] A few macrophages within this tissue were moderately to robustly positive for AIF1 (Figure 2 C). In a rat liver, a focal chronic lesion had expansion and effacement of sinusoids due to $AIF1^+$ macrophages and tissue remodeling (Figure 2 D).

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Table 1. Origins of tissue samples for AIF1 immunostaining

Species	Background	No. of animals	Sex	Age	Tissues evaluated
Mouse	CD1	2	F	10 mo	liver, spleen, lung
Mouse	129[a]	2	F	5 mo	liver, spleen, lung
Mouse	129[a]	2	M	5 mo	liver, spleen, lung
Mouse	C57BL/6[a]	2	M	4 mo	liver, spleen, lung
Mouse	C57BL/6[a]	2	F	4 mo	liver, spleen, lung
Mouse	C57BL/6[a]	2	F	10 mo	liver, spleen, lung
Mouse	C57BL/6[a]	2	M	10 mo	liver, spleen, lung
Mouse	C57BL/6J	2	F	17 mo	liver, spleen, lung
Mouse	C57BL/6J	2	M	17 mo	liver, spleen, lung
Mouse	BALB/c	1	F	11 mo	liver, spleen, lung
Mouse	BALB/c	1	M	11 mo	liver, spleen, lung
Rat	Fischer 344	1	M	6 mo	liver, spleen, lung
Rat	Sprague–Dawley	2	F	5 mo	liver, spleen, lung
Rat	WKY	2	F	9 mo	liver, spleen, lung
Rat	WKY	1	M	7 mo	liver, spleen, lung
Rat	WKY	1	M	9 mo	liver, spleen, lung
Rat	SHR	2	F	9 mo	liver, spleen, lung
Rat	SHR	1	M	7 mo	liver, spleen, lung
Rat	SHR	1	M	9 mo	liver, spleen, lung
Rat	WKY	1	M	2.5 mo	lung
Rat	WKY	1	M	2.5 mo	lung
Rat	WKY	1	M	2.5 mo	lung
Rat	SHR	1	M	2.5 mo	lung
Rat	SHR	1	M	2.5 mo	lung
Rat	SHR	1	M	2.5 mo	lung
Rat	LE	2	F	17 mo	liver, spleen, lung
Rat	LE	1	M	22 mo	liver, spleen, lung
Rat	LE	1	M	18 mo	liver, spleen, lung
Ferret	Wild type	2	F	6 mo	liver, spleen, lung
Ferret	Wild type	1	M	22 mo	liver, spleen, lung
Ferret	Wild type	1	M	11 mo	liver, spleen, lung
Pig	Wild type	1	F	30-36 h	liver, spleen, lung
Pig	Wild type	1	F	3 mo	liver, spleen, lung
Pig	Wild type	1	M	30-36 h	liver, spleen, lung
Pig	Wild type	1	M	24 h	liver, spleen, lung
Human	Wild type	1	F	16 y	lung
Human	Wild type	1	F	17 y	lung
Human	Wild type	1	M	5 y	lung
Human	Wild type	1	M	25 y	lung

[a]Animal was on the respective background with genetic modifications.

Because we observed robust AIF1 immunostaining in macrophages of the inflamed rat lung, we examined samples of noninflamed and inflamed human lung tissue to determine whether AIF1 immunostaining was similar between these conditions (Figure 3 A and B, respectively). In noninflamed lung (Figure 3 A), AIF1$^+$ macrophages had similar localization patterns (interstitium and alveolar macrophages) as in samples from animal models (Figure 1 B), but the inflamed lung had robust AIF1 immunostaining in intralesional macrophages and along airspaces (Figure 3 B).

We then tested AIF1 in a rat model of hypertension. Immunocytochemistry of alveolar macrophages obtained through bronchoalveolar lavage of SHR and WKY rats did not show any staining or morphologic differences (data not shown). However, AIF1 immunostaining (Figure 3 C and D) of WKY alveolar macrophages showed mild to moderate immunostaining in a diffuse cytoplasmic pattern, whereas SHR alveolar macrophages were often increased in size (cytoplasm and nuclei) with central pallor (that is, lack of immunostaining) in the relatively foamy cytoplasm. Evaluation of macrophages for morphologic evidence of activation and size revealed increases ($P < 0.05$) in both parameters in SHR compared with WKY rats (Figure 3 E, F). The unusual eccentric immunostaining of alveolar macrophages in the hypertensive rats may reflect early activation associated with the initiation of phagocytosis or membrane ruffling[34,35] or might represent different subtypes of macrophage activation.[18]

Discussion

We examined whether AIF1 could be used as a macrophage marker across species. Using an immunohistochemistry

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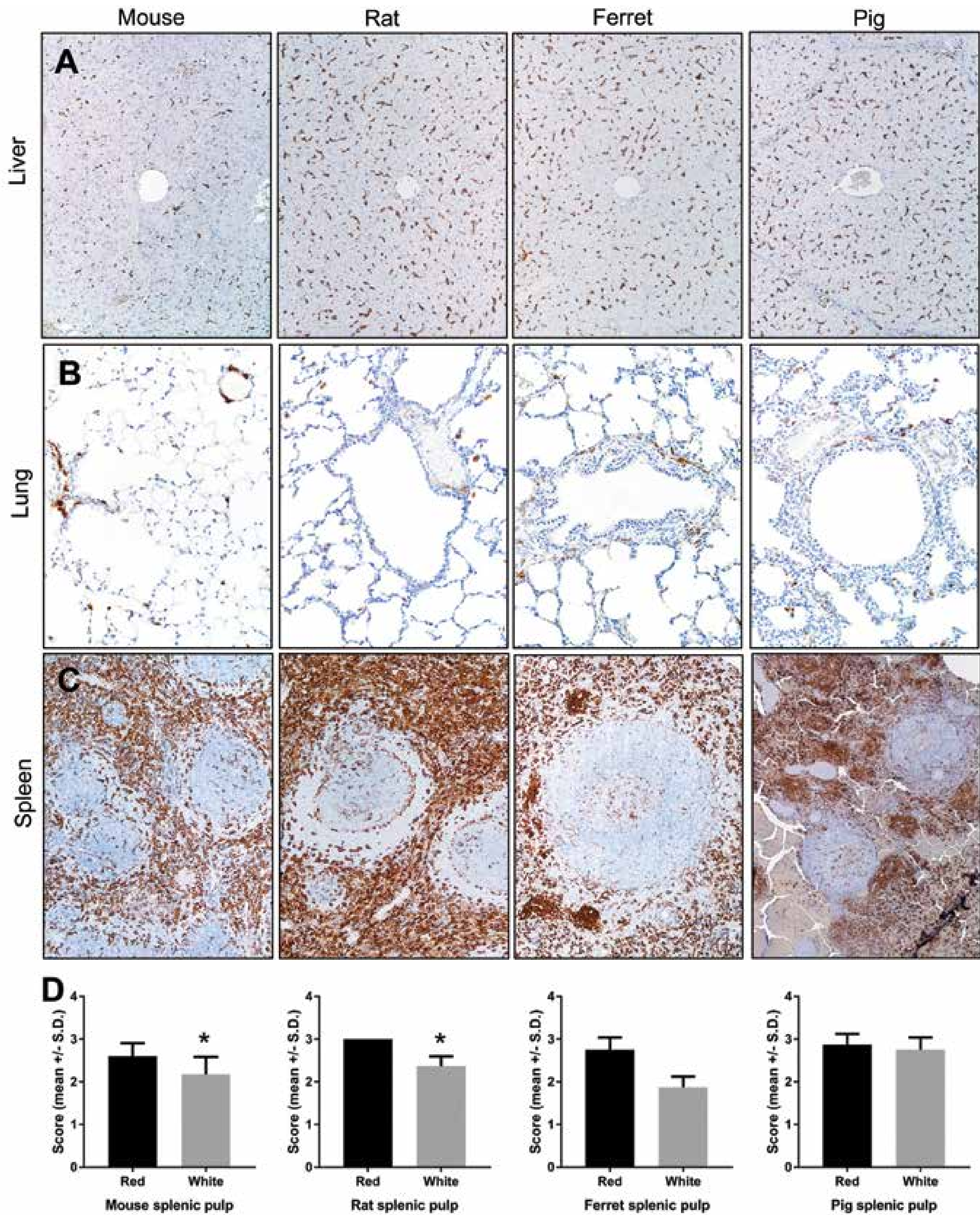

Figure 1. (A through C) AIF1 immunostaining (brown) in tissues and (D) scoring. (A) In liver, AIF1 immunostaining is seen in scattered interstitial cells consistent with Kupffer cells. (B) In lung, AIF1 immunostaining is moderate to robust in scattered interstitial macrophages, with weak to moderate staining in alveolar macrophages. (C) In spleen, AIF1 immunostaining in macrophages is prominent in red pulp, with fewer stained cells in the white pulp. DAB chromogen and hematoxylin counterstain; magnification, 100×. (D) Ordinal scoring and Wilcoxon matched-pairs signed rank analysis of AIF1 immunostaining intensity in splenic macrophages reveals more intense staining in red pulp than white pulp in mice (n = 20, P = 0.0002) and rats (n = 15, P < 0.0001); but differences in ferrets (n = 4) and pigs (n = 4) did not achieve statistical significance.

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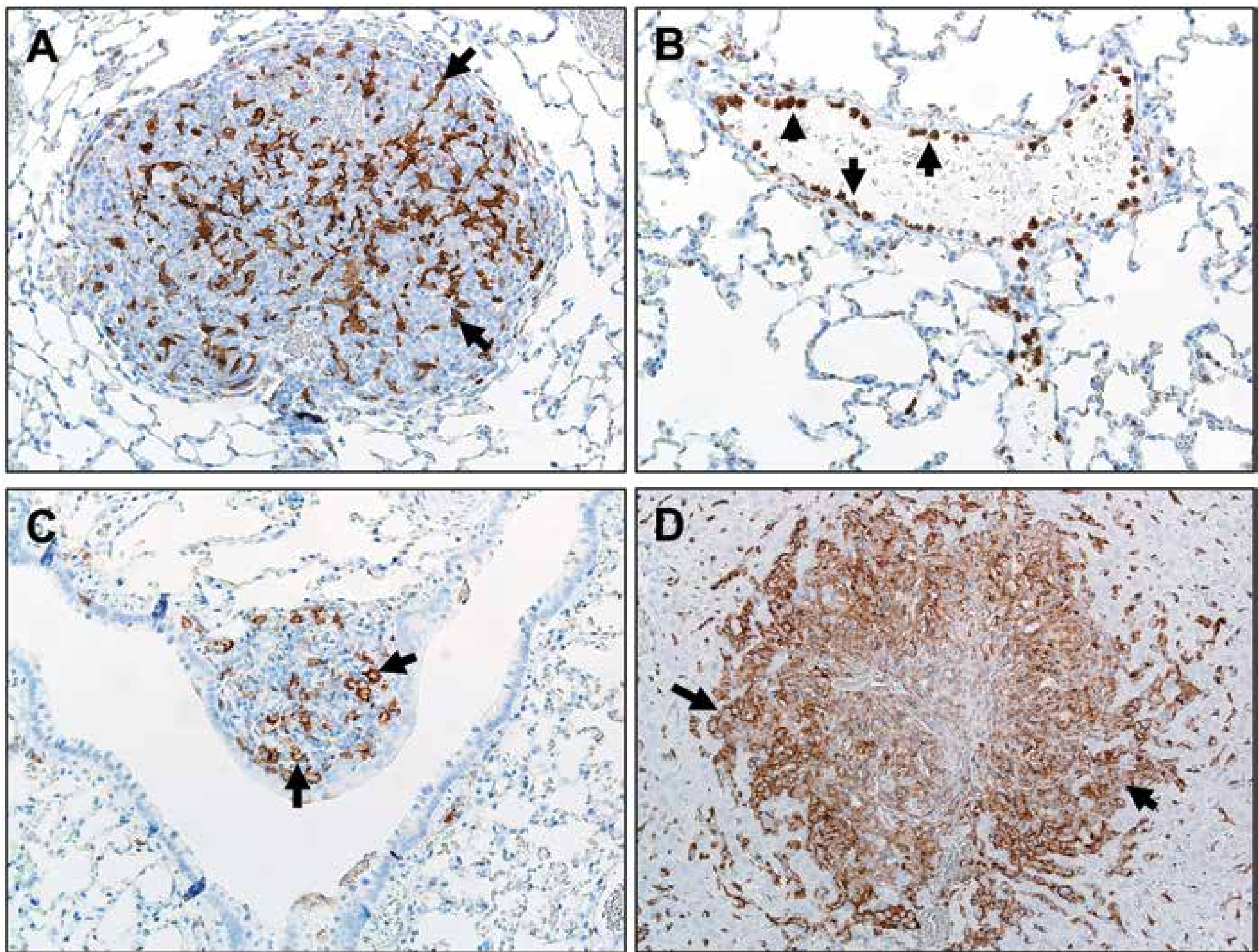

Figure 2. AIF1 immunostaining (brown color) in (A through C) lung and (D) liver. (A) Several AIF1⁺ macrophages (arrows) within a papillary adenoma of a mouse lung. (B) Several AIF1⁺ cells (arrows) marginated along the vessel wall in a rat lung. (C) Scattered AIF1⁺ macrophages (arrows) within inducible bronchus-associated lymphoid tissue localized at the junction of a branching airway in a rat lung. (D) Several AIF1⁺ macrophages (arrows) in a chronic lesion of a rat liver. DAB chromogen and hematoxylin counterstain; magnification: 200× (A through C), 100× (D).

technique validated in humans, we demonstrated appropriate anatomic immunostaining across 4 laboratory animal species and 3 organs, thus confirming our initial hypothesis. In addition, we had the opportunity to demonstrate AIF1 immunostaining of macrophages within several incidental tissue changes and pathologic lesions in sections. Finally, we tested AIF1 immunostaining in a rat model of hypertension and identified phenotypic differences in alveolar macrophages. Our combined results suggest that AIF1 is a useful marker for the identification of activated macrophages.

Historically, AIF1 has primarily been applied as a marker of microglia of the nervous system in multiple species.[6,8,21,23,41] In contrast, AIF1 has been used infrequently to detect macrophages in other tissues. For example, AIF1 expression has been observed in monocytic cell lines[20] and in activated macrophages after cardiac transplantation of rats and humans.[47,48] More recently, AIF1 has been used for macrophage identification in ferret inflammation,[10] rat uveitis,[9] pig lung,[29] mouse testis,[25] brain development of NHP,[46] histiocytic sarcoma of rabbits,[22] cutaneous disorders of dogs and cats,[38] activated microglia in medicinal leeches,[8] and peripheral blood monocytes in humans with rheumatoid arthritis.[37] Although these previous studies indicated that AIF1 might have the potential for macrophage identification across species, our current work demonstrates and validates the use of this marker in 4 common laboratory animal species. Of note, in many of the previous publications regarding this protein, AIF1 was called ionized calcium-binding adapter molecule 1 or even IFNγ-responsive transcript 1, but AIF1 is currently the preferred nomenclature for the marker (www.genenames.org).

Our present study has several limitations and advantages to acknowledge. One potential limitation is that we focused on only 3 tissues from 4 species of laboratory animals. As such, we cannot state with confidence that using the marker in other tissues or other laboratory animal species might be as successful in achieving similar sensitivity and specificity patterns. The selection of the evaluated tissues was advantageous, given that they frequently had macrophages in anatomically discreet regions, such that we were able to show not only positive immunostaining here but also lack of staining in sites and cells that we expected to be negative (for example, hepatocytes), thus further validating the immunohistochemistry technique. Another advantage is that we assessed multiple rodent strains in the study, thereby suggesting common utility across several rodent strains and genotypes. The selection of a polyclonal primary antibody that was validated in human tissue was advantageous and

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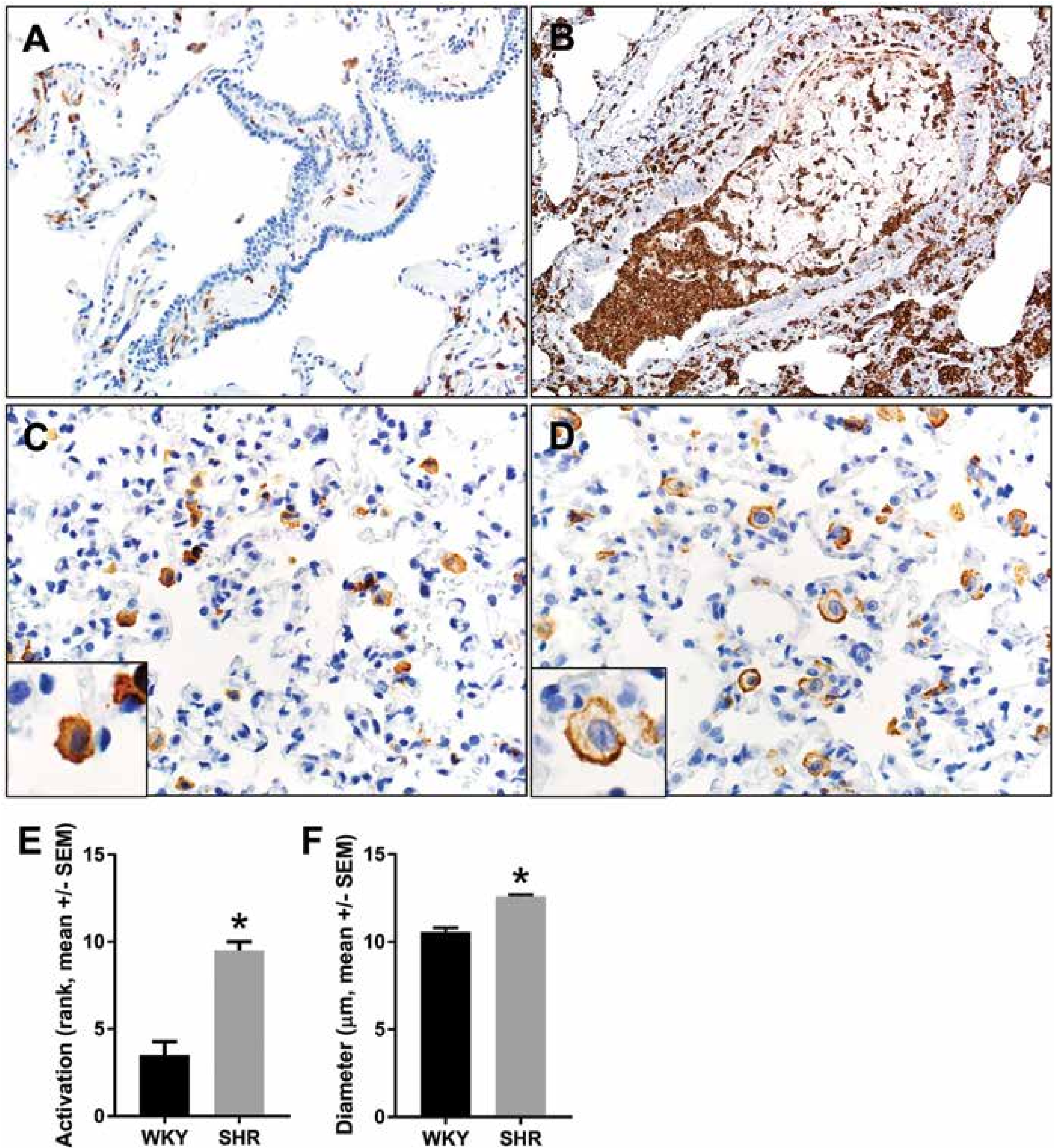

Figure 3. AIF1 immunostaining and scoring. (A and B) Immunostaining of AIF1⁺ macrophages in human lungs that (A) lacked inflammation or (B) had focal inflammation shows enhanced AIF1⁺ macrophages within and around airspaces. (C and D) Lung samples from (C) WKY rats had alveolar macrophages with relatively uniform moderate cytoplasmic AIF1 immunostaining. In contrast, (D) SHR alveolar macrophages were larger, with preferentially eccentric cytoplasmic AIF1⁺ immunostaining. DAB chromogen and hematoxylin counterstain; magnification: 200× (A), 100× (B), 400× (C and D). (E) Semiquantitative rank scores for features of macrophage activation (for example, enlarged nucleus, increased foamy cytoplasm), $P = 0.028$ (unpaired *t*-test) between rat lines. (F) Quantitative scoring of macrophage diameter, $P = 0.0016$ (unpaired *t*-test) between rat lines.

supported increased likelihood of marker detection across several species, and the required antibody dilution mitigated issues regarding nonspecific background staining. The prospective use of AIF1 in the same platform (that is, technique) across species offers cost savings to the research laboratory and facilitates increased importance and immediacy of translation for studies in animal models of human disease.

With the ever-widening application of genetically modified mice and other laboratory animal species, it is increasingly useful to have validated immunohistochemical markers for

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humans that function across laboratory animal species.[40,50] Mice likely will continue to be used extensively in translational research, because they provide a wide range of genetic models, have a short life span and high reproductive fecundity, and are inexpensive to house. However, some of the challenges and pitfalls in translating mouse research studies to humans have garnered increased attention recently. Most notable is the recognition that murine models may not accurately demonstrate the relevant human pathology (such as for cystic fibrosis, Alzheimer disease, and Huntington disease).[15,24,26,39,44,45,51] As such, because of promising advances in the genetic modification of nonmurine species, large animal models are increasing in use for many diseases, thus widening the scope of animal models.[27,42,43] The validated and common platform of the immunostaining protocol for AIF1 makes the marker more useful and attractive for animal models in translational research.

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