ARTICLE
Journal of Cellular Biochemistry 115:391–396 (2014)

Journal of Cellular Biochemistry

Specific In Situ Detection of Murine Indoleamine 2, 3-Dioxygenase

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ABSTRACT

Indoleamine 2,3-dioxygenase-1 (IDO1) catabolizes the essential amino acid tryptophan, acting as a modifier of inflammation and immune tolerance. Recent work has implicated IDO1 in many human diseases, including in cancer, chronic infection, autoimmune disorders, and neurodegenerative disease, stimulating a major surge in preclinical and clinical studies of its pathogenic functions. In the mouse, IDO1 is expressed widely but in situ detection of the enzyme in murine tissues has been unreliable due to the lack of specific antibodies that do not also react with tissues from animals that are genetically deficient in IDO1. Such probes are crucial to establish cellular mechanisms since IDO1 appears to act in different cell types depending on disease context, but reliable probes have been elusive in the field. In this report, we address this issue with the development of IDO1 monoclonal antibody 4B7 which specifically recognizes the murine enzyme in tissue sections, offering a reliable tool for immunohistology in preclinical disease models. J. Cell. Biochem. 115: 391–396, 2014.

KEY WORDS: INDOLEAMINE 2,3-DIOXYGENASE-1; TRYPTOPHAN; CANCER

Indoleamine 2,3-dioxygenase-1 (IDO1) is a heme-containing enzyme that catalyzes the rate-limiting step in tryptophan catabolism to *N*-formyl-kynurenine. The reduction in local tryptophan concentration and the production of immunomodulatory tryptophan metabolites contribute to the ability of IDO to modify inflammation and immunity [Mellor and Munn, 2008; Prendergast et al., 2011]. For example, IDO activity modulates the character of inflammatory responses in the tissue microenvironment to support carcinogenesis [Prendergast et al., 2010]. IDO suppresses the function of T effector cells, favors differentiation of T regulatory cells and is considered as a mediator of immune escape in cancer [Munn and Mellor, 2007; Prendergast, 2008; Cesario et al., 2011;]. In the mouse, genetic and pharmacological proofs have established that IDO1 drives carcinoma progression and the creation of a metastatic niche [Muller et al., 2005; Hou et al., 2007; Muller et al., 2008; Smith et al., 2012].

With the rapid increase of preclinical studies of IDO1 in mouse models of disease, one persistent deficiency has been the availability of reliable antibodies that can specifically detect the enzyme in murine tissues. Indeed, to our knowledge, there are no antibodies currently available that lack non-specific binding to tissues from IDO1-deficient mice, hampering reliable immunohistological analyses. This paper addresses this issue by describing the development and characterization of a monoclonal antibody (mAb) that specifically and reliably detects the IDO1 enzyme in mouse tissues.

MATERIALS AND METHODS

PEPTIDE SEQUENCE AND SYNTHESIS

For synthesis of peptides we selected epitopes that are surface-oriented and hydrophilic [Sugimoto et al., 2006]. We determined three regions on the IDO1 protein sequence that had good hydrophilicity as predicted by the Lasergene software (DNAStar, WI). We selected the epitope at the N-terminal sequence of the protein based on previous attempts to generate immunohistocompatible antibodies against murine IDO1 (Fig. 1A). The 3D structure of IDO1 (Fig. 1B) was modeled using the online server for I-TASSER (iterative threading assembly refinement) [Zhang et al., 2005; Zhang, 2008; Roy et al., 2010; Thomas et al., 2011]. Through these methods we chose a 20-mer peptide sequence derived from murine IDO1 amino acids 60–79 (Genbank sequence NP_032350.1, designated muIDO1$_{60-79}$ as indicated by the red line in Fig. 1A) to synthesize and conjugate to KLH (GenScript, Piscataway, NJ) for use in mouse immunization. As a species control for screening IDO1 mAb, we also synthesized a peptide derived from the analogous primary sequence in human IDO1 designated huIDO$_{58-75}$.

IMMUNIZATIONS AND HYBRIDOMA GENERATION

All procedures involving the use of animals were approved by the Lankenau Animal Care and Use Committee. *Ido1*−/− BALB/c mice

Grant sponsor: NIH; Grant number: R01 CA109542.

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Manuscript Received: 9 September 2013; Manuscript Accepted: 10 September 2013
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 30 September 2013
DOI 10.1002/jcb.24674 • © 2013 Wiley Periodicals, Inc.

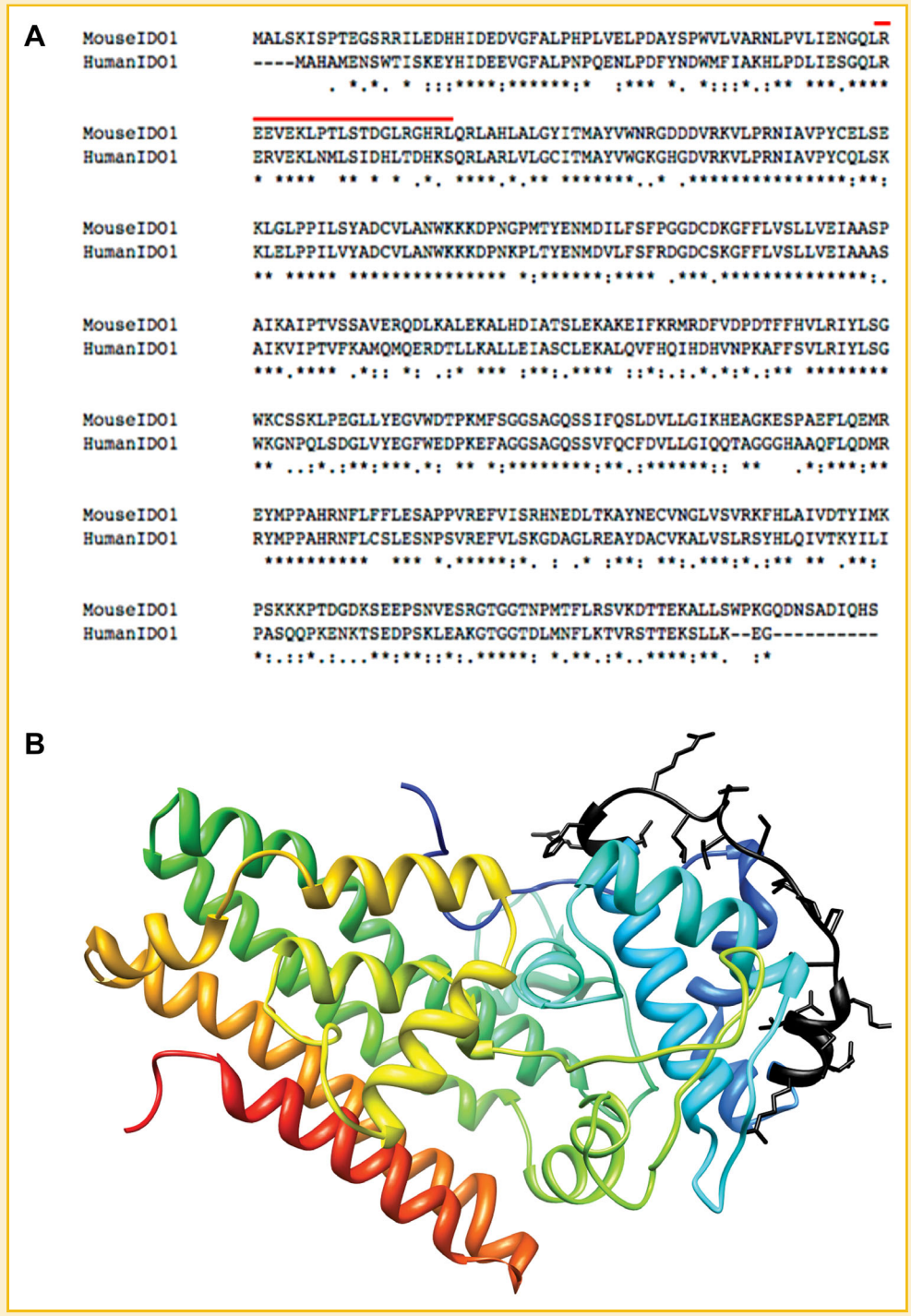

Fig. 1. Selected immunogen aligned to the IDO amino acid sequence. The IDO1 peptide selected for mouse immunization to raise antibodies was aligned to (A) the full-length primary amino acid sequences of the mouse and human enzymes and (B) a three-dimensional structural model of mouse IDO1. Amino acid sequence of the mouse IDO1 peptide (highlighted in black) used to generate anti-mouse IDO1 antibodies.

were immunized with the KLH-muIDO1$_{60-79}$ conjugate in complete Freund's adjuvant. The initial injection was followed by one boost in incomplete Freund's adjuvant and a final boost in PBS. Hybridomas were generated by standard methods [Koprowski et al., 1979] and cloned in methyl cellulose using a vendor's protocol (Stem Cell Technologies, Vancouver, BC, Canada).

ELISA ASSAYS

To evaluate the specificity of the antibodies secreted by the hybridomas obtained, we coated 96-well dishes (MaxiSorp, Nunc, Denmark) with an aqueous solution of the IDO1$_{60-79}$ peptide (2 μg/ml) at 4°C overnight. After washing with PBS, dishes were blocked 1 h at 4°C with 5% fetal calf serum in PBS/0.1% Tween20 and then incubated 1 hr with hybridoma

supernatents at room temperature. At the end of this incubation, dishes were washed with PBS and incubated 1 h with a mouse-specific secondary antibody linked to horseradish peroxidase (HRP). Antibody conjugates on the dishes were detected by addition of substrate 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) and a 10–20 min incubation at room temperature. Optical densities were measured at 410 nm on an ELISA plate reader (Synergy 2, Bio-Tek Instruments, Winooski, VT). All assays, post hybridoma screening were performed in triplicate wells of the dish and the average values were calculated. For determination of antibody subclasses, the same procedure was performed except that the secondary antibodies used were HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, IgG2c, IgG3, or IgM antibodies (Southern Biotech, Birmingham, AL) added at a dilution of 1:300.

WESTERN ANALYSIS

Wild-type (WT) and *Ido1*−/− C57BL6/J male mice administered LPS were euthanized 24 h after injection as described [Muller et al., 2010]. Organs collected at necropsy were homogenized in a polytron blender in the presence of RIPA buffer containing protease and phosphatase inhibitors. Tissue lysates were centrifuged and protein concentrations determined. Equal protein for each sample (typically 30 μg/lane) were fractioned by SDS–PAGE and blotted to Immobilon-NC membranes (Millipore). After blocking, the blots were incubated at 4°C overnight with IDO1 monoclonal antibody 4B7 followed by a 1 h incubation with an HRP-conjugated goat anti-mouse secondary antibody. Blots were washed, developed with HYGLO Quickspray chemiluminescent HRP reagent (Denville Scientific, NJ) and exposed to Hyblot autoradiography film (Denville Scientific).

TISSUE IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY

Organs from WT and *Ido1*−/− C57BL6/J male mice administered LPS were obtained as before and stored at −80°C. Frozen tissues were embedded in OCT and sections prepared with a cryomicrotome on glass slides were warmed to room temperature 5 min and then fixed 10 min with ice-cold acetone. Slides were dried 5 min and tissue boundaries marked with a PAP pen. Tissue sections were then rehydrated 20 min with PBS, treated 5 min with ice-cold 0.3% hydrogen peroxide, washed 20 min with PBS and blocked 30 min with 5% normal goat serum in PBS/0.1% Tween20. After blocking, tissues were treated 45 min at room temperature with the primary antibody diluted in the blocking buffer. Following three 5 min washes with PBS/0.1% Tween20, tissues were incubated 30 min at room temperature with the HRP-conjugated secondary antibody diluted in blocking buffer. After washing three times as before, sections were given one final wash in PBS and then developed by incubation with DAB substrate in PBS for 10 min before washing one last time with PBS and then deionized water. To visualize histology, tissue sections were treated briefly with hematoxylin and rinsed with deionized water. Dehydration procedures consisted of treating the sections with 70% ethanol (5 min), 95% ethanol (5 min), 100% ethanol (5 min) twice, followed by treatment for 5 min with xylene twice. Slides were mounted using VectaMount mounting medium (Vector Laboratories, CA) for visual microscopy.

For immunofluorescence microscopy, the procedure used was the same except that the secondary antibody used was conjugated to the fluorescent dye Cy3 instead of HRP (1:500 dilution). The goat anti-mouse Cy3-conjugated antibody was incubated 30 min at room temperature in the dark, followed by three 5 min washes with PBS/0.1% Tween20. After a final 5 min wash in PBS, slides were mounted using Vectashield with DAPI mounting medium (Vector Laboratories) and viewed under an epifluorescence microscope (Zeiss, Germany).

RESULTS

MONOCLONAL ANTIBODY CHARACTERIZATION

We selected a 20-mer peptide from the murine IDO1 sequence for immunization of *Ido1*−/− mice as described in the Materials and Methods Section (Fig. 1A,B). Eight hybridomas generated by myeloma fusion using standard techniques were selected based on the reactivity of the secreted mAb to recognize the muIDO1$_{60–79}$ peptide immunogen in the KLH conjugate used for vaccination (1A10, 4B7, 6F5, 10C6, 10D9, 11A12, 12G2, and 12H11). A non-reactive hybridoma, clone 1B2, was used as a non-specific control in our experiments. On the basis of ELISA and western blot analyses, the hybridomas 4B7 and 10D9 were found to offer the most attractive mAb candidates, of which 4B7 was determined subsequently to be the best overall for tissue staining. ELISA assays were used initially to evaluate mAb specificity. Dishes coated with a human IDO1 peptide derived from the analogous sequence (huIDO1$_{56–75}$) were probed with the mAb secreted by 4B7 and 10D9 that exhibited positivity for the murine IDO1 peptide. Neither of these antibodies cross-reacted with the human peptide, indicating that the 4B7 and 10D9 mAbs were specific for the muIDO1$_{60–79}$ peptide (Fig. 2A). Using isotype-specific secondary antibodies, we determined that these mAbs were each of the IgG1 isotype (Fig. 2B). Although both were positive in the ELISA assay against the muIDO1$_{60–79}$ peptide, we found that only 4B7 mAb could detect full-length IDO1 protein in mouse tissue extracts by western blotting. For example, 4B7 mAb detected murine IDO1 in colon and epididymis of animals treated with bacterial LPS, an inducer of IDO1 expression, whereas 4B7 mAb did not detect any band in tissues from similarly treated mice that were nullizygous for the Ido1 gene (Fig. 3). These results showed that 4B7 mAb specifically recognized murine IDO1.

4B7 MAB SPECIFICALLY DETECTS IDO1 IN MOUSE TISSUES

A lack of probes for reliable in situ detection of murine IDO1 prompted us to compare staining by 4B7 of tissues derived from WT and *Ido1*−/− mice. Using indirect immunofluorescence to examine acetone-fixed frozen tissue sections, we evaluated specific mAb staining in epididymis, colon, heart, and liver (Fig. 4A–D). Notably, while producing a positive signal in WT tissues, 4B7 mAb produced no detectable signal in *Ido1*−/− tissues under the same conditions employed. Consistent with a previous report [Britan et al., 2006], we observed the highest levels of IDO1 in epididymis compared to the other tissues examined, reinforcing the notion that 4B7 mAb can specifically detect murine IDO1 protein. Extending these observations, we also used 4B7 mAb to evaluate the immunohistochemical expression of IDO1 protein in lung metastases of 4T1 breast tumor-bearing mice, a setting where we recently established a critical functional role for IDO1 in generating a metastatic niche [Smith

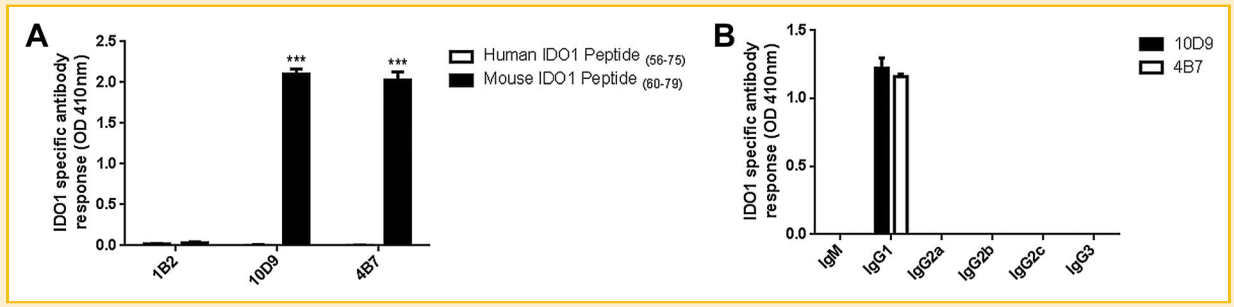

Fig. 2. mAb specificity for murine IDO1. A: mAb secreted by the hybridomas 4B7 and 10D9 but not 1B2 reacted with the muIDO1$_{60-79}$ peptide but did not cross-react with the huIDO1$_{56-75}$ peptide derived from the analogous region of human IDO1. B: Antibody isotyping indicates both 4B7 and 10D9 mAb are IgG1.

et al., 2012]. Notably, while metastases in the lungs of WT mice stained positively, consistent with a positive modifier effect of IDO1 on malignant progression in this setting, no staining was detected in a lung metastasis derived from *Ido1*−/− mice (Fig. 4E), where such lesions only occur more rarely [Smith et al., 2012].

To establish that 4B7 mAb would be effective for use in immunohistochemistry of mouse tissue, we also employed standard methods to compare staining of frozen sections of colon tissue obtained from WT and *Ido1*−/− mice treated with LPS. An intense positive stain was detected in the colonic crypts of WT animals but not in the same structures of *Ido1*−/− mice (Fig. 5). Thus, 4B7 mAb appears to be specific for in situ detection of IDO1 in mouse tissues by immunofluorescence or bright-field microscopy. Overall, we conclude that 4B7 mAb offers an useful novel probe for in situ analysis of murine IDO1 expression, addressing a need for the growing number of preclinical studies of IDO function in development, immune physiology, and pathophysiology.

DISCUSSION

IDO1 is an emerging therapeutic target for cancer, infection, autoimmunity, neurodegenerative disorders, and other diseases characterized by chronic inflammation and pathological immune suppression [Prendergast et al., 2011]. Effective mAb probes for human IDO1 are available for clinical immunohistochemistry,

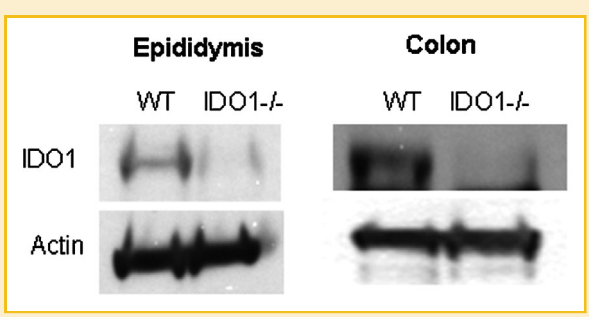

Fig. 3. Specificity of 4B7 mAb in Western analysis of mouse tissue extracts. An IDO1-specific band was detected in epididymis and colon extracts derived from WT mice but not from *Ido1*−/− mice.

including clone 10.1 mAb commercialized from our group that has been used widely [Muller et al., 2005]. However, mAb probes for in situ detection of murine IDO1 have been more problematic. In our hands, the available published and commercial mAbs tested to date demonstrate strong non-specific staining of *Ido1*−/− tissues. The lack of an IDO1 specific probe limits the impact of work in preclinical mouse models used to study disease etiology, development, treatment, and prevention, an important concern given the tractable nature of IDO as a target for medical intervention and the recent advance of IDO inhibitors into clinical trials.

We used *Ido1*−/− mice to establish the reliable specificity of 4B7 mAb for tissue staining applications. 4B7 mAb was specific for the murine enzyme and did not cross-react with human IDO1. It was active in ELISA and western blotting applications as well as immunomicroscopy, thus providing an all-around probe of IDO1 in mice. One report used mIDO-48, a commercially available mAb, to characterize the immunohistochemical expression of IDO1 in paraffin-embedded tissues [Dai and Zhu, 2010]. While we found that mIDO-48 can specifically recognize murine IDO1, we observed non-specific staining of *Ido1*−/− tissues (unpublished observations). In this study, we used immunofluorescence microscopy to detect IDO1 expression in fixed frozen tissues, a protocol expected to be more sensitive than immunohistochemistry and light microscopy for detection. Importantly, we did not detect cross-reacting signal in tissue from *Ido1*−/− mice. Our data suggests that 4B7 is more specific than mIDO-48 for tissue staining applications. We observed staining patterns in epididymis and colon, both expressing relatively high levels of IDO1, which were similar to that reported by Dai and Zhu [2010] using mIDO-48. Thus, the selectivity of 4B7 may be most useful in other tissues characterized by lower levels of IDO1 protein expression.

Two unique variables in generating 4B7 mAb were the novel peptide epitope and the use of an *Ido1*−/− BALB/c host for immunization. The hydrophilic muIDO1$_{60-79}$ peptide is not fully conserved within the human sequence and lies on the surface of the protein structure. Use of the *Ido1*−/− host for immunization may have altered the response to what would otherwise have been, a self-antigen. Whether either of these factors were important in obtaining the 4B7 mAb is not clear, but the use of genetically deficient mice for raising mAb to self-antigens is relatively unstudied and may offer an option in similar situations where useful antibodies have been difficult to obtain.

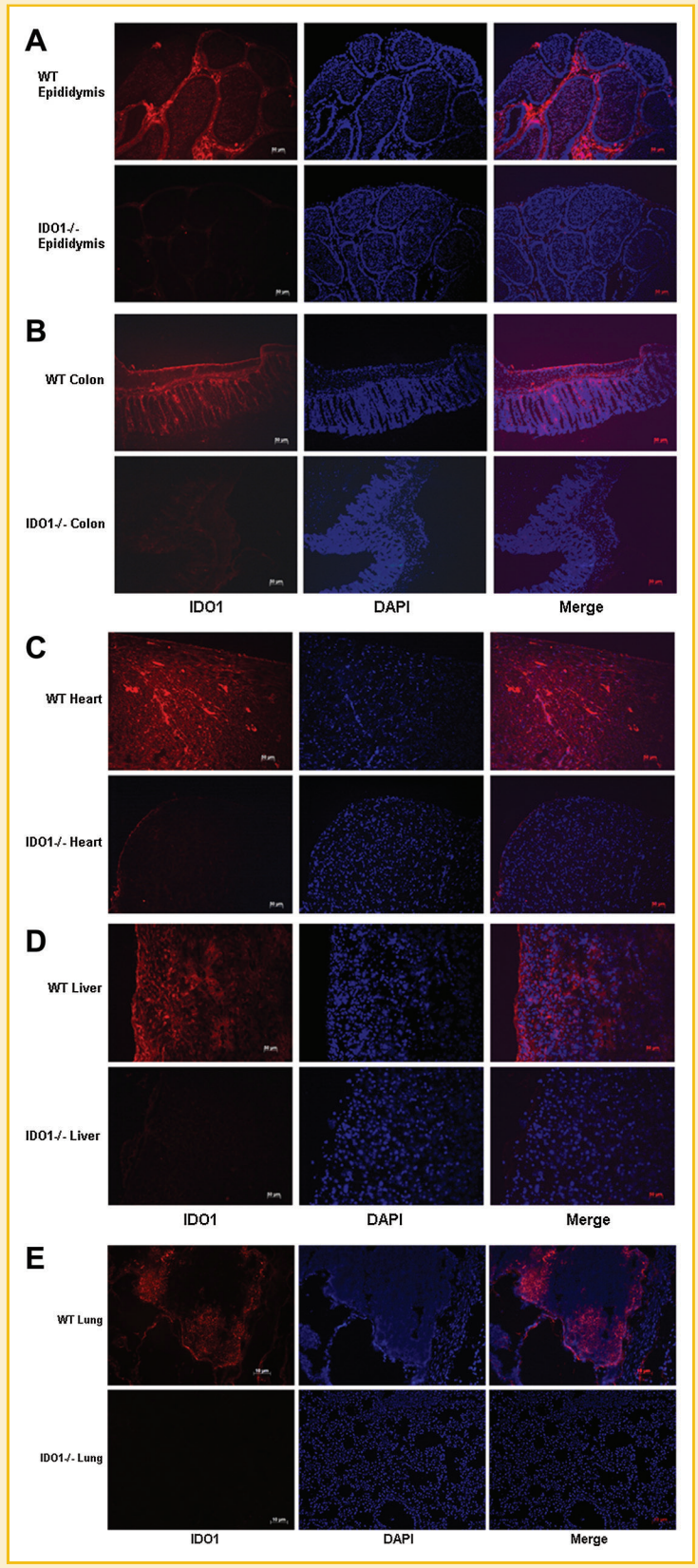

Fig. 4. Specificity of 4B7 mAb for immunofluorescence of mouse tissue. Acetone-fixed frozen tissues were positively stained when derived from WT mice but not *Ido1−/−* mice. A: Epididymis. B: Colon. C: Heart. D: Liver. E: Lung metastases derived from the 4T1 model of breast cancer [Smith et al., 2012].

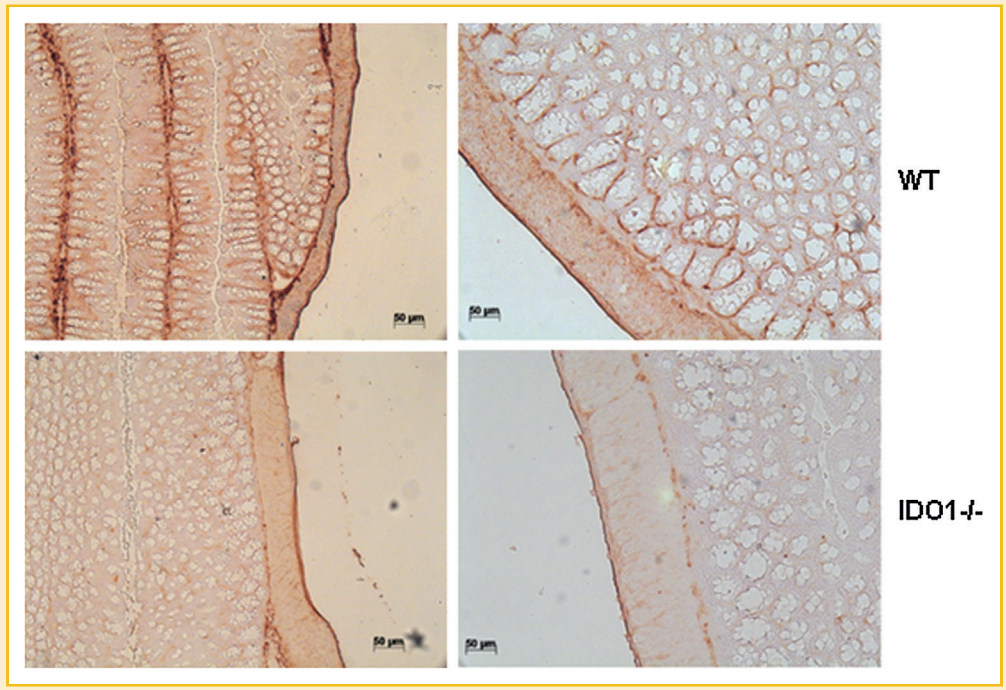

Fig. 5. Specificity of 4B7 mAb for immunohistochemistry of mouse tissue. Acetone-fixed frozen sections of colon tissue were positively stained when derived from WT mice but not *Ido1−/−* mice.

ACKNOWLEDGMENTS

We thank Courtney Smith for providing lung tissues for immunomicroscopy, and Jennifer Mulgrew and Gwen Guillard for sectioning the tissue samples. This work was supported in part by NIH grant R01 CA109542 (G.C.P.).

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