Histochemical characteristics of regressing vessels in the hyaloid vascular system of neonatal mice: novel implication for vascular atrophy

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ABSTRACT

The hyaloid vasculature constitutes a transitory system nourishing the internal structures of the developing eye, but the mechanism of vascular regression and its cell biological characteristics are not fully understood. The present study aimed to reveal the specificity of the hyaloid vessels by a systematic immunohistochemical approach for marker substances of myeloid cells and the extracellular matrix (ECM) in neonatal mice. Macrophages immunoreactive for F4/80, cathepsin D, and LYVE-1 gathered around the vasa hyaloidea propria (VHP), while small round cells in vascular lumen of VHP were selectively immunoreactive for galectin-3; their segmented nuclei and immunoreactivities for Ly-6G, CD11b, and myeloperoxidase indicated their neutrophilic origin. VHP possessed thick ECM and a dense pericyte envelope as demonstrated by immunostaining for laminin, type IV collagen, integrin β1, and NG2. The galectin-3^+ cells loosely aggregated with numerous erythrocytes in the lumen of hyaloid vessels in a manner reminiscent of vascular congestion. Galectin-3 is known to polymerize and form a complex with ECM and NG2 as well as recruit leukocytes on the endothelium. Observation of galectin-3 KO mice implicated the involvement of galectin-3 in the regression of hyaloid vasculature. Since macrophages may play central roles including blocking of the blood flow and the induction of apoptosis in the regression, galectin-3^+ neutrophils may play a supportive role in the macrophage-mediated involution of the hyaloid vascular system.

Keywords: galectin-3, LYVE-1, integrin, hyaloid vessels, regression, immunohistochemistry

1. Introduction

The hyaloid vascular system, consisting of the vasa hyaloidea propria (VHP) and the tunica vasculosa lentis (TVL), is a transiently existing vessel network important for nourishment of the developing lens and maintenance of the primary vitreous. This vascular system in mice starts to form at embryonic day 10.5 (E10.5) and becomes fully developed by E13.5 (Smith et al., 2002). It regresses and then replaces developing permanent retinal vessels; in the mouse, VHP and TVL initiate the regression after birth and largely disappear around postnatal day 16 (Ito and Yoshioka, 1999; Brown et al., 2005). The regression is associated with apoptosis of endothelial cells/pericytes and phagocytosis of their apoptotic bodies by macrophages (Jack, 1972; Mitchell et al., 1998; Taniguchi et al., 1999). The exact regulatory mechanism responsible for the involution of hyaloid vessels is poorly understood; there are, however, some contributing factors: an increased expression of apoptosis factors, the down-regulation of angiogenic growth factors such as VEGF, and cessation of the blood flow (for review, Saint-Geniez and D'Amore, 2004; Hegde and Srivastava, 2017).

Macrophages are known to gather around regressing blood vessels of VHP (Ito and Yoshioka, 1999; Taniguchi et al., 1999). Electron microscopic studies have demonstrated the phagocytosis of vessel-derived debris by macrophages (Taniguchi et al., 1999). Using macrophage-deficient mice, it has been shown that macrophages play an essential role in the regression of the hyaloid vascular system (Lang and Bishop, 1993). Histochemically, Zhang et al. (2010) detected a topographical association of macrophages with VHP using an antibody against LYVE-1, which was originally identified as a receptor molecule for hyaluronan. LYVE-1 is structurally homologous to another hyaluronan receptor, CD44, which is widely expressed on leukocytes, dendritic cells, and tumor cells (Lesley et al.,

1994). For further characterization of the macrophages in regressing hyaloid vessels, we tried to use another unique marker of macrophages, galectin-3 (Sato and Hughes, 1994; Liu et al., 1995; Sano et al., 2003), in the immunostaining of whole mount preparations of developing murine eyes.

Galectin is one of the animal lectins which recognize β-galactoside of glycoconjugates and contribute to various biological functions, such as cell differentiation, migration, and apoptosis (Yang et al., 2008). Among fifteen galectin subtypes, galectin-3 is the only one classified as a chimera-type and possesses a unique ability for self-assembly. In addition to monocytes/macrophages, this endogenous lectin-like protein is contained in several blood cells including neutrophils, eosinophils, and mast cells (Liu, 2000, 2005; Dumic et al., 2006). Although galectin-3 is present predominantly in the cytoplasm, it is released from cells by a non-classical mechanism, due to a lack of the signal sequence or transmembrane domain in the protein (Hughes, 1999; Dumic et al., 2006). Extracellular galectin-3 binds to cell surface oligosaccharides, thereby exerting various functions represented by apoptosis (Suzuki et al., 2008; Zhuo et al., 2008) and cell adhesion (Sato and Hughes, 1992; Inohara et al., 1996; Kuwabara and Liu, 1996). Notably, Galectin-3 forms a molecular complex with NG2 chondroitin sulfate proteoglycan and extracellular matrixes (ECM) such as integrin (Ochieng et al., 1998; Fukushi et al., 2004) and laminin (Kuwabara and Liu, 1996; Hughes, 2001) to promote cell-ECM attachment. Support for the involvement of galectin-3 in physiologic and pathologic consequences of blood vessels has been attributed to thrombogenesis and neovasculogenesis. Leukocytes expressing galectin-3 attached to the vein wall in a thrombosis model of mice and the expression levels of monomers to trimmers were up-regulated in loco (DeRoo et al., 2017).

When we stained the hyaloid vessels of neonatal mice using several anti-galectin-3 antibodies, the LYVE-1-immunoreactive macrophages were found to be negative, but cells accumulating in the lumen of hyaloid vessels were selectively immunoreactive for galectin-3. In contrast, the developing blood vessels in the retina contained few galectin-3-expressing cells in the vascular lumen. The present study examined the detailed characteristics of the galectin-3-expressing cells in the hyaloid vessels and suggests their possible involvement in the regression of the hyaloid vasculature.

2. Methods

2.1. Animals and tissue sampling

Pregnant ddY mice and CD1 mice were supplied by Japan SLC (Shizuoka, Japan) and Charles River Laboratories Japan (Yokohama, Japan), respectively. The eyeballs of E17.5 embryos and neonates at postnatal days 1, 3, 5, 7, 9, 15, and 20 (ddY strain) were mainly used in the present study. The generation of galectin-3-deficient mice of the CD1 strain was described previously (Hsu et al., 2000). Samples from the galectin-3 KO mice were obtained at postnatal days 9, 15, and 20. Mice were sacrificed by the intraperitoneal injection of an overdose of pentobarbital sodium (Schering Plough Animal Health, the Netherlands). The eyeballs were enucleated and fixed for 2 h in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4. The retina with vitreous was spread by cutting into fours, and the lens were isolated from eyeballs under a dissecting microscope.

All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

2.2. Immunohistochemistry

After immersion in 0.01 M phosphate buffered saline (PBS) containing 0.3% Triton-X100, the whole mount preparations in glass bottles were pre-incubated with a normal donkey serum. For double or triple immunostaining, they were incubated for 3 days with a mixture containing two or three antibodies from the following list: goat anti-mouse galectin-3 antibody (AF1197; R&D Systems, Minneapolis, MN), rabbit anti-mouse LYVE-1 antibody (11-034; AngioBio, Del Mar, CA), rabbit anti-myeloperoxidase antibody (PA5-16672; ThermoFisher Scientific, Rockford, IL), goat anti-mouse cathepsin D antibody (AF1029; R&D Systems), rat anti-mouse F4/80 antibody directly labeled with or without Alexa Fluor 647 (BM8; BioLegend, San Diego, CA), rat anti-mouse integrin β1(CD29) antibody (MAB2405; R&D Systems), rabbit anti-laminin antibody (ab11575; abcam, Cambridge, UK), rabbit anti-NG2 chondroitin sulfate proteoglycan antibody (AB5320; Millipore/Chemicon International, Temecula, CA), rabbit anti-type IV collagen antibody (ab19808; abcam), rat anti-mouse/human CD44 antibody labeled with PE (IM7; affymetrix/ThermoFisher Scientific), rat anti-mouse Ly-6G/Ly-6C antibody (RB6-8C5; Novus Biologicals, Littleton, CO), rat anti-mouse CD68 antibody labeled with or without Alexa fluor 488 (FA-11; affymetrix and BioLegend), rat anti-mouse/human CD11b antibody labeled with or without Alexa Fluor 488 (M1/70; BioLegend), rat anti-mouse CD31 antibody (MEC 13.3; BD Pharmingen, Franklin Lakes, NJ), rabbit anti-MCT1 antibody (MCT1-Rb-Af900; Frontier Institute, Ishikari, Japan), and guinea pig anti-GLUT1 antibody (GLUT1-GP-Af610; Frontier Institute). The antigen-antibody reactions were detected by incubation of 6 h with Cy3-conjugated donkey anti-goat, anti-rat, or anti-rabbit IgG antibody; Cy5-conjugated donkey anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, PA); Alexa Fluor 488-conjugated donkey anti-rat,

anti-rabbit, or anti-guinea pig IgG antibodies (Invitrogen/ThermoFisher Scientific). Stained samples were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (Fluoview FV300; Olympus, Tokyo, Japan). The specificity of immunoreactions on sections was confirmed according to a conventional procedure, including absorption tests.

For statistic evaluation, galectin-3^+ cells and LYVE-1^+ macrophages were counted in five independent areas of VHP, 600×800 μm in size, randomly chosen from 5 eyes at each stage and the numbers of galectin-3^+ cells and LYVE-1^+ cells were counted. Data are expressed as mean ± standard errors of the mean (SEM). Statistical comparisons between stages were evaluated by one-way analysis of variance (ANOVA) with Sidak's multiple comparisons test using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Values in the graphs represent the mean ± SEM and $P < 0.05$ was regarded as significant.

2.3. Silver-intensified immunogold method for electron microscopy

Some of the fixed tissues containing the VHP were dipped in 30% sucrose solution overnight at 4^oC, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections 12 μm in thickness were mounted on poly-L-lysine-coated glass slides. They were pretreated with normal donkey serum for 30 min, incubated with the goat anti-galectin-3 antibody (1 μg/mL) overnight, and subsequently reacted with rabbit anti-goat IgG covalently linked with 1-nm gold particles (Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmicated, dehydrated, and directly embedded in Epon. Ultrathin sections were prepared and stained with an aqueous solution of uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

3. Results

3.1. Galectin-3-expressing cells gather in the hyaloid vessels.

As reported by Zhang et al. (2010), LYVE-1-immunoreactive cells of round or irregular shapes gathered around the blood vessels of the vasa hyaloidea propria (VHP) and the tunica vasculosa lentis (TVL) in fetuses and neonates. They were immunostained positively with F4/80 antibody (Fig. 1a), a reliable marker antibody of murine macrophages, and also immunoreactive for cathepsin D (Fig. 1b), one of the lysosomal degradation enzymes, thus strongly indicating their macrophagic origin. They were distributed only along the VHP/TVL but completely absent in other regions without hyaloid vessels (Fig. 1c). They appeared to attach or be juxtaposed to the blood vessels of VHP with short distances (Fig. 1b). Interestingly, the β1 integrin filaments, like a tangled web, arrested LYVE-1^+ cells and loosely bound them with vessels (Fig. 1d). When we stained whole mount preparations using the galectin-3 antibody at Day 5 and Day 7, the immunoreactivity for galectin-3 was found only in small round cells residing inside the vessels of VHP/TVL and was not detectable in the LYVE-1^+ macrophages outside the vessels (Fig. 1e). VHP and TVL detectable by GLUT1 antibody (Kishimoto et al. 2016) contained a great number of galectin-3^+ cells, but retinal vessels without GLUT1 immunoreactivity possessed few galectin-3^+ cells in the lumen (Fig. 1f and Supporting Fig. 1a). These galectin-3-expressing cells in the hyaloid vasculature were rare before birth (E17.5) and in Day 1 neonates (Fig. 2a); all of them, if present, resided outside the vessels. Their number rapidly increased inside the vessels at Day 3, starting at proximal branches of VHP/TVL with a large diameter (compare Fig. 2b and Supporting Fig. 1a). After postnatal 5 days, the galectin-3^+ cells were densely distributed in the vascular lumen throughout the VHP and TVL (arrows in Fig. 2c, d). In contrast, numerous

F4/80$^+$ LYVE-1$^+$ macrophages persisted even before birth and showed a considerably consistent frequency of occurrence throughout the perinatal period. When the frequency of galectin-3$^+$ cells was compared with that of LYVE-1$^+$ cells in the VHP, the ratios of galectin-3$^+$ cells to LYVE-1$^+$ cells were less than 10% at Day 1 and Day 3, while they were more than 50% at Days 5 and 7 (Fig. 3). The numbers of LYVE-1$^+$ cells per area significantly decreased in Days 5 and 7, possibly due to the enlargement of VHP.

Galectin-3$^+$ cells displayed immunoreactivities for CD68 and CD11b, markers of a monocyte/granulocyte lineage (yellow colored cells in Fig. 4a, b), but not for F4/80 in tripled immunostaining. Galectin-3$^+$ cells definitely corresponded to cells immunoreactive for Ly-6G, a marker of neutrophils (Fig. 4c). Most of the round galectin-3$^+$ cells were about 8 μm in diameter, being comparable in size to erythrocytes, and possessed segmented nuclei. They often displayed irregular shapes, occasionally extending long cytoplasmic processes within the lumen of vessels (Supporting Fig. 2a, b). The galectin-3$^+$ cells contained a small number of myeloperoxidase-positive granules in the cytoplasm (Fig. 4d). Electron-microscopically, gold particles showing the existence of galectin-3 were localized in the cytoplasm with spotted dense immunoreaction along the cell surface (Fig. 5a, b). These characteristics suggest that galectin-3$^+$ cells are in the lineage of neutrophils.

CD44, a hyaluronan receptor, is broadly distributed in a variety of immune cells including granulocytes, macrophages, and lymphocytes (Lesley et al., 1994). The antibody against CD44 stained the galectin-3$^+$ cells in the VHP of neonatal eyes but not so LYVE-1$^+$ macrophages outside the VHP (Fig. 5c). Interestingly, LYVE-1 expressed by the perivascular macrophages is known as another hyaluronan receptor

(Banerji et al., 1999). The staining characteristics are reminders that the vitreous is a representative space extremely rich in hyaluronan.

3.2. Blood vessels of VHP are rich in extracellular matrix.

Next, we stained the hyaloid vascular system using a panel of antibodies against ECM and related molecules to find other characteristics of this unique vascular system. By this, the regressing vessels of VHP were found to possess thick ECM along endothelial cells. Immunostaining for laminin (Fig. 6a), type IV collagen, and integrin β1 (Supporting Fig. 1b, c) all demonstrated the existence of a thick basement membrane in blood vessels of VHP and also TVL. The immunoreactivities for these ECM were much more intense than those in the retinal vascular system. Moreover, the immunostaining of ECM intensely labeled pericytes associated with vessels—to be precise, the basement membrane surrounding the pericytes (Fig. 6a). The pericytes of VHP were densely distributed and showed an intense immunoreactivity for NG2 (Fig. 6b and Supporting Fig. 1d, e), which is a marker substance of pericytes, especially activated ones (Ozerdem et al., 2001; Fukushi et al., 2004). During the regression of VHP, some vessels were reduced in diameter to thin, threadlike strands, as clearly displayed by laminin immunostaining (Fig. 6c). ECM and pericytes with intense immunoreactivities for each of marker substances were still associated with the threadlike vascular remnants. The vascular remnants frequently contained fragmented endothelial cells with CD31 immunoreactivity in dilated and shut portions (Fig. 6c).

3.3. Congestion of blood cells including galectin-3^{+} cells in TVL

To evaluate blood flow in the hyaloid vessels, we tried to detect red blood cells

by immunostaining for MCT1. MCT1, a major subtype of monocarboxylate transporter (MCT), is expressed in red blood cells, especially in the neonatal period (Supporting Fig. 2c, d) (Takebe et al., 2011; Iwanaga and Kishimoto, 2015). In these samples, red blood cells appeared to congest in vessels of TVL (Fig. 6d), but such a picture was never obtained in the retinal vessels. Characteristically, the aggregated red blood cells intermingled with many galectin-3^+ cells.

Finally, to examine the involvement of galectin-3 in the regression process of hyaloid vessels, we assessed the hyaloid vasculature in galectin-3 knockout (KO) mice at Day 9, Day 15, and Day 20, when the regression of hyaloid vessels had become advanced. For an overall observation of blood vessels, we examined TVL using an antibody against GLUT1, which was intensely expressed in the endothelium of the hyaloid vascular system (Kishimoto et al., 2016). Samples from galectin-3 KO mice did not contain galectin-3^+ cells in any regions of eyes. TVL of galectin-3 KO mice maintained a considerable number of blood vessels at the posterior face of the lens, as compared with those from wild type mice at Day 9 (Fig. 7) and Day 15 (Supporting Fig. 3a, b). The deficiency of galectin-3 did not change essentially development of the retinal vasculature (Supporting Fig. 3e, f). Collectively, these results indicate the significant involvement of galectin-3 in the regression of hyaloid vessels.

4. Discussion

4.1 Characteristics of galectin-3-expressing leukocytes

It was a unique finding that galectin-3-expressing cells were accumulated in the VHP and TVL of the hyaloid vascular system but not in vessels extending within the developing retina. What type of cells are the galectin-3^+ cells showing an intense affinity to the hyaloid vessels? The galectin-3^+ cells as well as perivascular

macrophages were positive for CD68, which is a marker of monocyte/macrophage lineage. The galectin-3^+ cells were also immunoreactive for CD11b and Ly-6G, the latter being specific to neutrophils. Other immunostaining results concerning F4/80, LYVE-1, and cathepsin D indicate that the galectin-3^+ cells lacking in these immunoreactivities differ from the extra-vascular macrophages with a high degrading capacity. In developing stages of the eye, $LYVE^+$ $F4/80^+$ macrophages appeared numerous even before birth in contrast to galectin-3^+ cells, which started to increase in number around postnatal 3 days. Electron-microscopically, the cytoplasm of galectin-3^+ cells was not occupied by specific granules of granulocytes, unlike typical mature granulocytes. However, the present study confirmed the existence of myeloperoxidase-positive granules, though not abundant, in the cytoplasm. Our double staining of galectin-3 and myeloperoxidase revealed their reverse intensities: granulocytes with a weak galectin-3 immunoreactivity showed an intense myeloperoxidase reactivity, and vice versa (data not shown). At present, the galectin-3-positive cells may be identifiable as an immature or special type of neutrophils.

The exact reason why the hyaloid vessels recruit galectin-3-expressing cells in the lumen is unknown. However, CD44 expressed by all galectin-3^+ cells is a receptor for hyaluronan extremely rich in the vitreous. Macrophages surrounding the hyaloid vessels and residing freely in the vitreous also expressed another hyaluronan receptor, LYVE-1 (Zhang et al., 2010), as confirmed by the present study. LYVE-1 may function as scaffold for fixing macrophages in the ECM of several tissues (Zheng et al., 2014). Thus, both the galectin-3^+ neutrophils and LYVE-1^+ macrophages possess an intense affinity to the hyaluronan-rich vitreous, and the two hyaluronan receptors may help with their recruitment and residence in the hyaloid vascular system. In addition to the affinity to hyaluronan, the expression of CD44 on

galectin-3^+ cells may assist their binding to the endothelium, since CD44 is a ligand of E-selectin that is abundantly expressed at the endothelial surface.

Galectin-3 functions as a chemoattractant molecule for macrophages (Sano et al., 2000; Gittens et al., 2017) and plays a role of opsonins in enhancing the phagocytic clearance of apoptotic cells but not viable cells by macrophages (Karlsson et al., 2009). In final stages of regression, released galectin-3 may be involved in drawing of macrophages and subsequent engulfment of apoptotic cells. Galectin-3 null mice displayed a deficiency in the accumulation of macrophages and neutrophils at inflammatory sites (Colnot et al., 1998; Hsu et al., 2000; Nieminen et al., 2008).

4.2 Characteristics of regressing hyaloid vessels

One of characteristics of hyaloid vessels is the abundant existence of some ECM components. Immunostaining for laminin, type IV collagen and integrin β1 demonstrated a thicker basement membrane in VHP than those in the retinal blood vessels. This finding is consistent with earlier ultrastructural observations showing thick and multilaminated basement membrane of the VHP (Jack, 1972; Balazs, 1975) and TVL (Latker and Kuwabara, 1981).

Numerous macrophages are distributed around the capillaries of hyaloid vessels in developing eyes (Zhang et al., 2010). They play an important role in the vascular regression of eyes, especially the induction of apoptosis and clearance of atrophic vessels (Lang and Bishop, 1993; Diez-Roux et al., 1999; Lobov et al., 2005). The filamentous network immunolabeled with the integrin β1 antibody was first documented in the present study; the morphological features suggest that it may function, at least partially, as a scaffold for moving macrophages. Although their significance is not fully disclosed, “the integrin web” may correspond to filamentous remnants observed under scanning electron microscope in the late

postnatal period of rats (Latker and Kuwabara, 1981). Failure of the fatal (or neonatal in the case of rodents) vasculature to undergo programmed involution results in persistent hyperplastic primary vitreous (PHPV), also known as persistent fetal vasculature (PFV). An evaluation of various gene knockout mice has proposed that integrins are most responsible for the congenital ocular malformations (Hegde and Srivastava, 2017). These authors focused on the important function of integrins as major cell-signaling centers and initiators of multiple signaling cascades. In the present study, we confirmed the endothelial integrin β1 to be a component of abundant ECM associated with VHP as well as the integrin web to serve as a scaffold of perivascular macrophages. These findings suggest the essential role of integrin β1 in mediating direct cell attachment and communication among cell elements associated with regressing hyaloid vessels.

Another immunostaining for NG2, which is a reliable marker of pericytes, displayed a dense distribution of immunoreactive cells in the hyaloid vessels. Furthermore, the NG2 immunoreactivity in the pericytes of VHP was more intense than in those of the retinal vessels, being a sign of active conditions of pericytes, since NG2 functions as a cell surface receptor for binding to ECM and for cross-talking to endothelial cells and interaction with growth factors (Ozerdem et al., 2001; Fukushi et al., 2004). It is generally believed that the detachment of pericytes induces neovascularization, namely the budding of vascular tips, implying that the dense distribution of pericytes negatively controls vascular development. Some vessels of VHP after neonatal day 7 became very thin—like paper strings, and the lumina were often occluded by cellular debris derived from endothelial cells. Although triggers of vascular involution are unknown, there is a possibility that the dense distribution of contractile pericytes with an elevated expression of NG2 strangles the blood vessels in the advanced stage of regression, resulting in the final

involution of hyaloid vascular system.

4.3 Possible involvement of galectin-3 in the initiation of vascular regression

Galectin-3 is a unique subtype of galectins that oligomerizes on the cell surface, resulting in various functions such as cell activation and cell adhesion. Galectin-3 oligomerizes at the surface of neutrophils and eosinophils to mediate the recruitment and firm adhesion of leukocytes to the endothelium (Sato et al., 2002; Nieminen et al., 2007; Rao et al., 2007). On the other hand, released galectin-3 has an ability to bind to endothelial cells to form a complex with NG2 and integrin β1, namely NG2/galectin-3/ECM complex, on the cell surface (Fukushi et al., 2004). The present study demonstrated the characteristic accumulation of galectin-3-expressing cells and upregulation of NG2 and ECM components as mentioned above, suggesting their intimate molecular relationship on the endothelial surface of regressing hyaloid vessels. Galectin-3 oligomerization may be important for clustering NG2 to permit more effective activation of integrin β1 (Fukushi et al., 2004) and might be involved together with a galectin-3 ligand, CD98, in the process of integrin activation (Hughes, 2001; Krzesla and Lipinska, 2004). Reportedly, β1 integrins but not β2 integrins have been implicated, via leukocyte-expressed galectin-3, in the recruitment and adhesion of neutrophils (Werr et al., 1998; Johnston and Kubes, 1999) and eosinophils (Rao et al., 2007) on endothelial cells. Taken together, these findings show it is likely that galectin-3 can control cell recruitment and the adhesion of circulating leukocytes through the modulation of integrin functions.

Accumulation of blood cells containing numerous galectin-3^+ cells was frequently observed in the VHP and TVL, being reminiscent of vascular congestion. Galectin-3^+ neutrophils may attach to the endothelium of VHP and TVL via a

complex formation of NG2/galectin/ECM to reduce blood circulation, finally effecting vascular atrophy. The involution of the hyaloid vasculature has been explained by cessation of the blood flow (Meeson et al., 1996) and the vasoconstriction of the proximal hyaloid vessels (Browning et al., 2001). Our observations of galectin-3 KO mice indicated some reduction in the regression of TVL. However, the reduction of regression became unclear at Day 20 (Supporting Fig. 3c, d), since it may be overcome by macrophages. Macrophages alone can induce the programmed regression of hyaloid vessels through the WNT pathway towards adjacent vascular endothelial cells (Lang and Bishop, 1993; Lobov et al., 2005). Thus, galectin-3^{+} neutrophils may play a supportive role in the macrophage-mediated regression of hyaloid vasculature.

In contrast to their inhibiting the blood supply, there is another possibility that galectin-3-expressing cells accumulate in the hyaloid vessels under ischemic conditions for neovasculogenesis. Galectin-3 induces the differentiation of endothelial cells in vitro and stimulates angiogenesis in vivo (Nangia-Makker et al., 2000; Wan et al., 2011). The angiogenetic effects of galectin-3 contradict our conclusion showing the involvement of galectin-3 in vascular atrophy. Overall, this newly discovered information concerning the regressing hyaloid vessels could provide new insight to the mechanisms underlying vascular atrophy or angiogenesis.

Declaration of interest

The authors have no competing interests to declare.

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Figure legends

Fig. 1 LYVE-1- and galectin-3-immunoreactive cells in the vasa hyaloidea propria (VHP) of neonatal mice. All LYVE-1-expressing cells react with antibodies against F4/80 (a) and cathepsin D (b), showing a merged color (yellow). In Fig. 1b, blood vessels are stained weekly red with CD31 antibody. Double staining for LYVE-1 and integrin β1 displays a restricted localization of LYVE-1-positive macrophages in the VHP region (c). At a high magnification of a different region from the same sample (d), LYVE-1-positive macrophages (red in color) are arrested by an integrin β1 meshwork like a tangled web and connected with vessels (v). Triple staining for LYVE-1, galectin-3, and CD31 shows that galectin-3-positive small round cells are localized within blood vessels of VHP (arrows) and free from LYVE-1 immunoreactivity (e). Dense distribution of galectin-3-immunoreactive cells is seen in tufts of GLUT1-immunoreactive VHP vessels but not in retinal vasculature indicated by asterisks (f). Bars 100 μm (a, c, f), 50 μm (b, e), 20 μm (d)

Fig. 2 Numerical changes of galectin-3-immunoreactive cells associated with VHP vessels from Day 1 to Day 7. At Day 1, galectin-3^+ cells are scarce and localized outside the vessels. Intravascular galectin-3^+ cells (arrowheads) do appear—though sparsely—at Day 3, while they start to increase at proximal parts of VHP around the optic disc (see Supporting Fig. 1a). At Day 5 and Day 7, VHP vessels indicated by arrows contain many galectin-3^+ cells in lumina of the vessels, and they are comparable in number to LYVE-1^+ macrophages residing outside the vessels. Blood vessels are stained weekly red with CD31 antibody. Bars 100 μm

Fig. 3 Frequency of the occurrence of galectin-3-expressing cells from E17.5 to

postnatal Day 7. Galectin-3-positive cells and LYVE-1-positive macrophages were counted in five independent areas (n=5) of 600×800 μm chosen randomly from five eyes at each stage. Data are expressed as mean ± standard errors of the mean (SEM). Values with different letters show a significant difference of $P < 0.05$.

Fig. 4 Expression of marker substances in galectin-3-containing cells. Galectin-3^+ cells express CD68 (a), CD11b (b), and Ly-6G (c). For CD68, extra-vascular macrophages (arrows) are also immunoreactive, showing a merged color. Most galectin-3^+ cells contain a small number of myeloperoxidase-positive granules (d). Bars 20 μm

Fig. 5 Immunoelectron microscopy for galectin-3 and double staining indicating expression of CD44 and galectin-3. In the immunogold method of VHP (a, b), gold particles showing the existence of galectin-3 label intravascular cells with an irregular nucleus or segmented nucleus (N). Gold particles gather at the periphery of the cytoplasm. Triple staining of galectin-3, LYVE-1 and CD44 (c) shows complete matching of immunoreactivities for galectin-3 and CD44 at a cellular level but separate immunolabeling with LYVE-1. Arrows indicate LYVE-1^+ macrophages. E: endothelium Bars 1 μm (a, b), 20 μm (c)

Fig. 6 Extracellular matrix of VHP vessels and congestion of blood cells in TVL. Immunostaining of laminin labels the thick basement membrane together with pericytes (arrows in a). Pericyte-specific NG2 immunoreactivity becomes intense in regressing vessels, where the CD31-positive endothelium is fragmented (arrows in b). At Day 9, some vessels of VHP are reduced in diameter to form thin,

threadlike strands, which are still intensely labeled for laminin (arrows in c-1). Asterisks indicate the retinal vasculature. At a higher magnification, debris of endothelial cells with CD31 immunoreactivity is trapped (arrows in c-2). In pictures for dual detection of erythrocytes and galectin-3^{+} cells in TVL, blood congestion is seen throughout the blood vessels of TVL, where erythrocytes are stained green with MCT1 antibody (d). Bars 250 μm (d-1), 100 μm (c-1), 50 μm (c-2), 20 μm (a, b, d-2)

Fig. 7 Posterior views of the lens at Day 9 in wild type (a) and galectin-3 KO mice (b). Arrows indicate regressed portions of TVL visualized with GLUT1 antibody. Regression of TVL in galectin-3 KO mice appears to be reduced to a certain degree. Images of double staining with LYVE-1 for macrophages are shown in Fig. 7a-2 and b-2. Representative images are shown from three cases for wild type and KO mice. Bars 100 μm

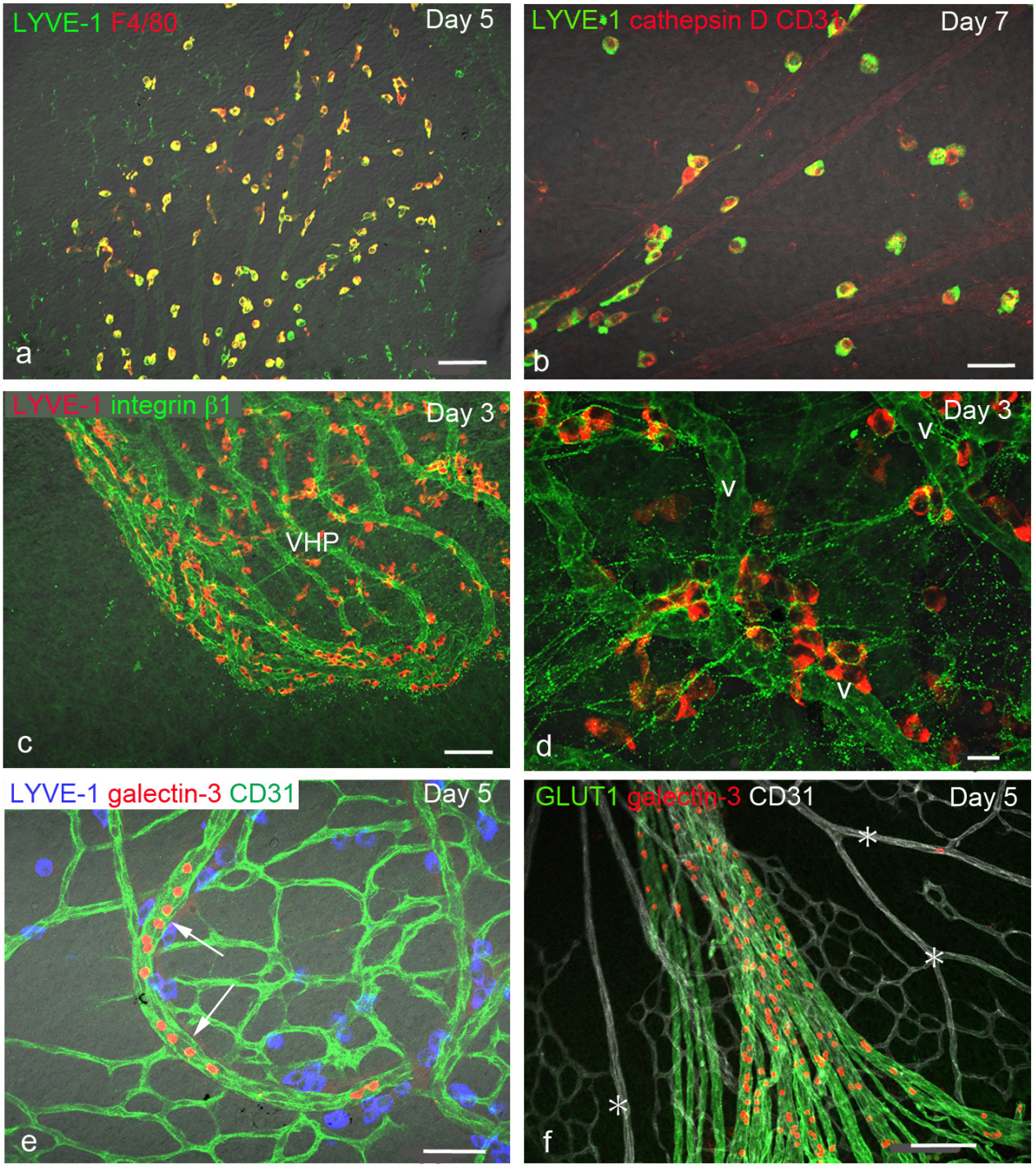
LYVE-1 F4/80
Day 5
a
LYVE-1 cathepsin D CD31
Day 7
b
LYVE-1 integrin β1
Day 3
VHP
c
Day 3
v
v
v
d
LYVE-1 galectin-3 CD31
Day 5
e
GLUT1 galectin-3 CD31
Day 5
f

Figure 1

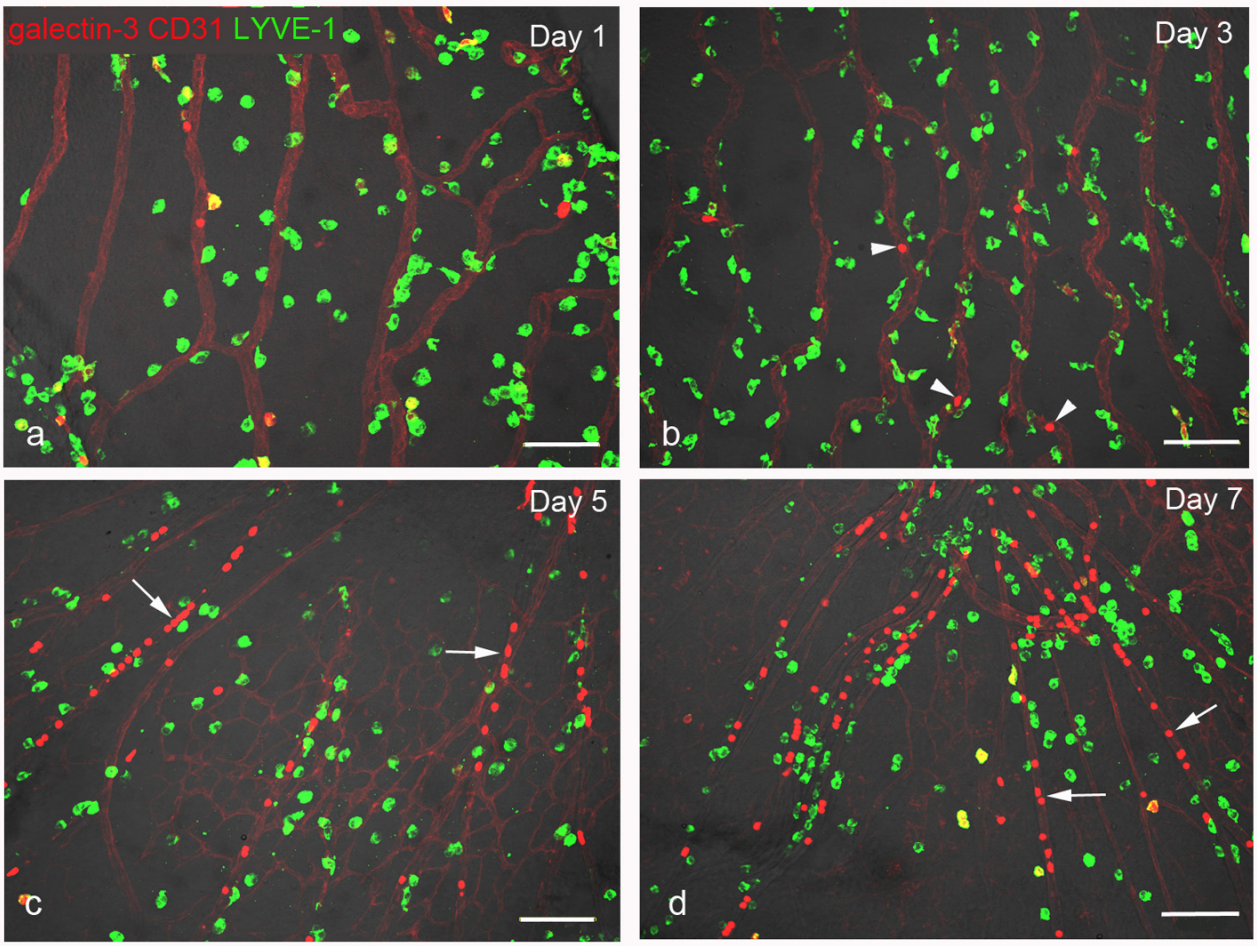

Figure 2

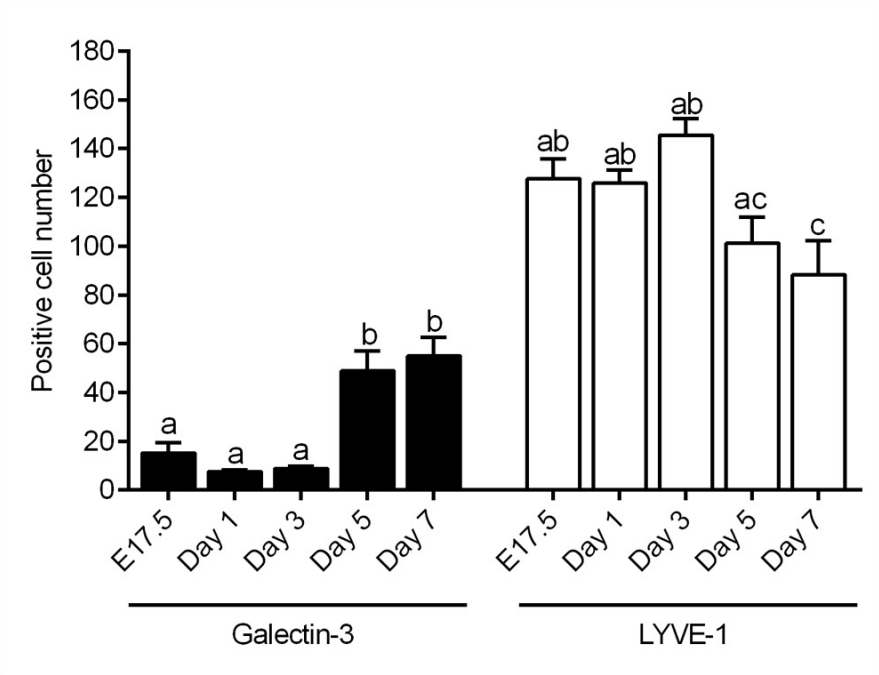
Positive cell number
180
160
140
120
100
80
60
40
20
0
a
a
a
b
b
ab
ab
ab
ac
c
E17.5
Day 1
Day 3
Day 5
Day 7
E17.5
Day 1
Day 3
Day 5
Day 7
Galectin-3
LYVE-1

Figure 3

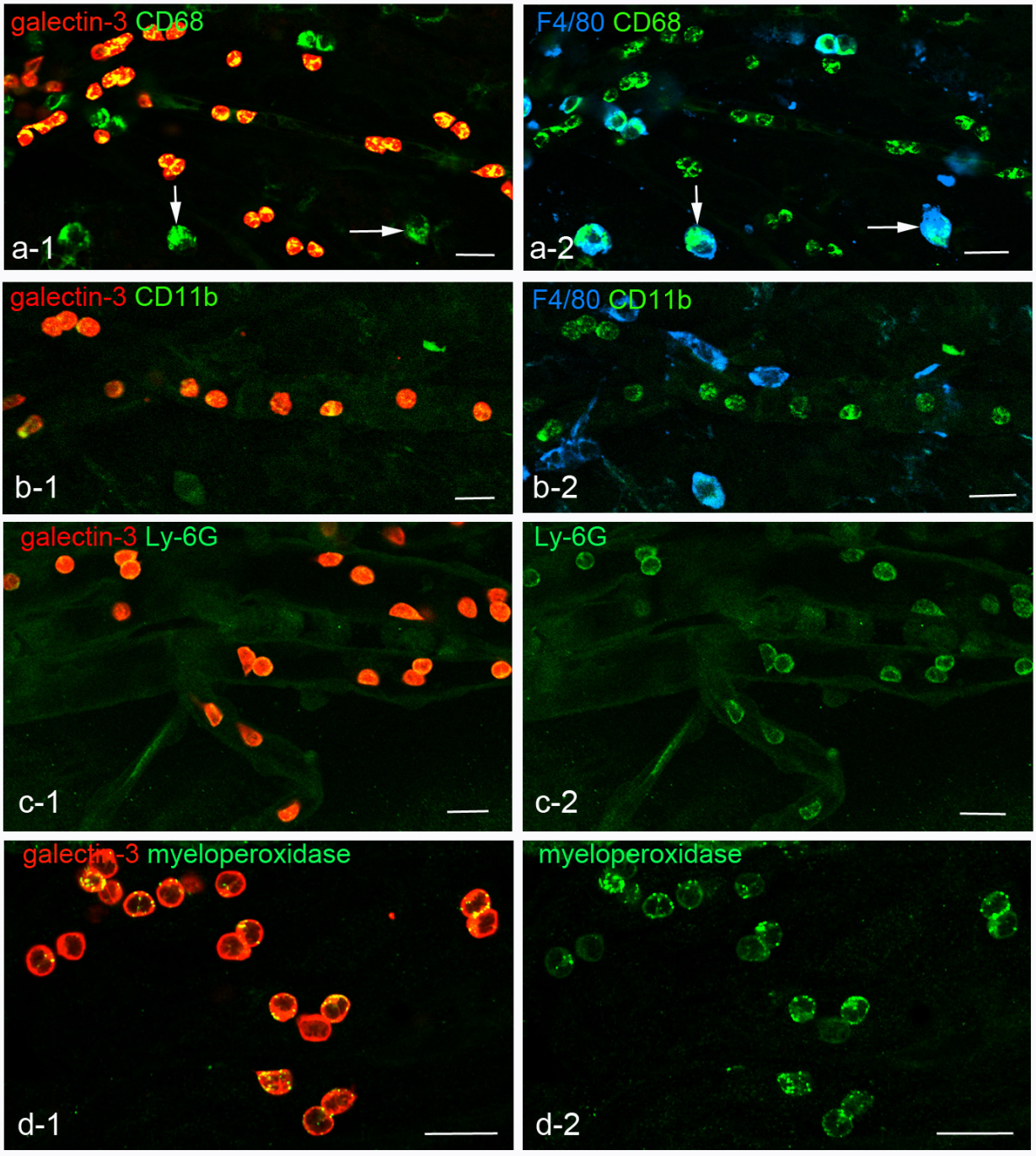
galectin-3 CD68
a-1
F4/80 CD68
a-2
galectin-3 CD11b
b-1
F4/80 CD11b
b-2
galectin-3 Ly-6G
c-1
Ly-6G
c-2
galectin-3 myeloperoxidase
d-1
myeloperoxidase
d-2

Figure 4

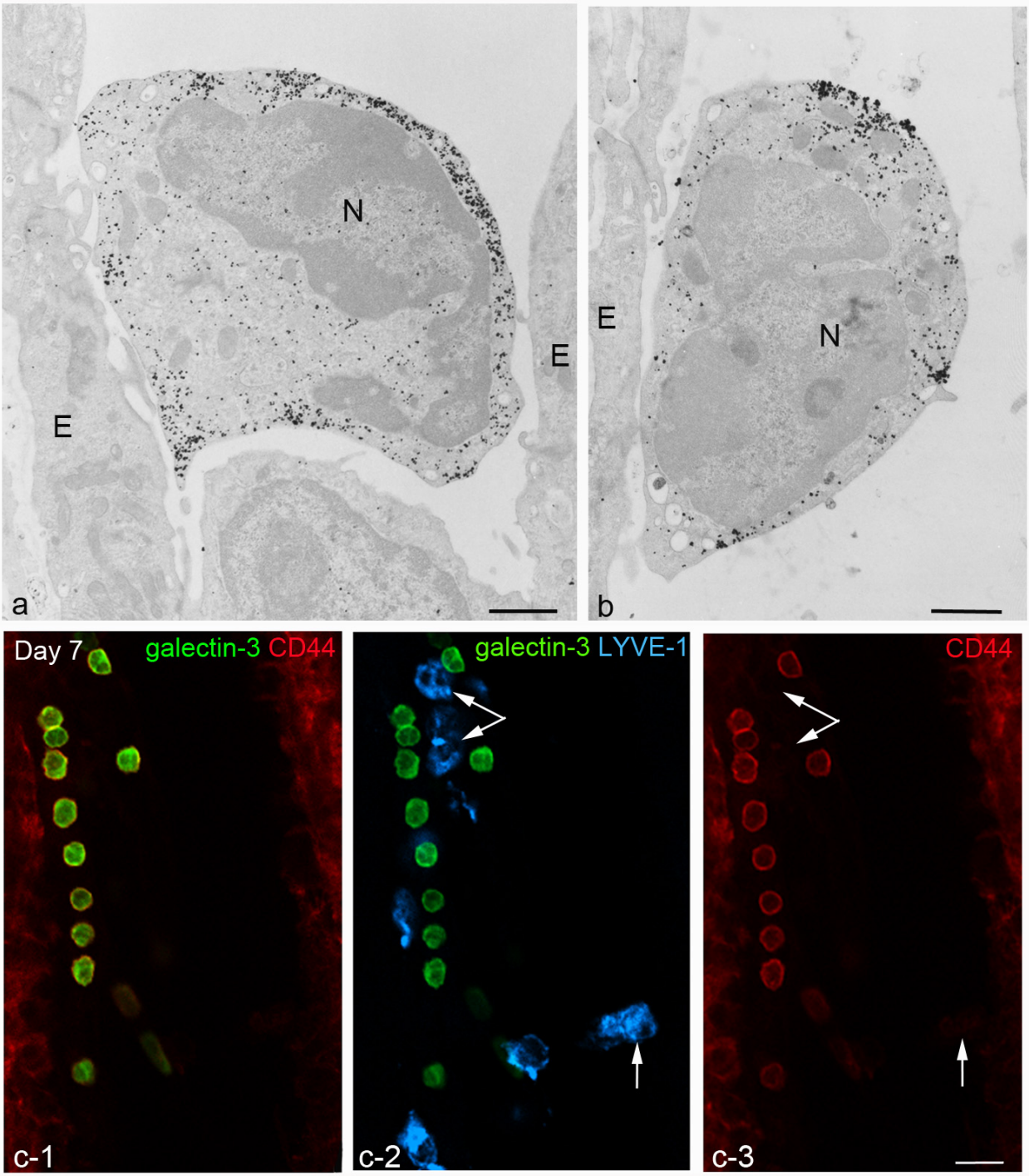

Figure 5

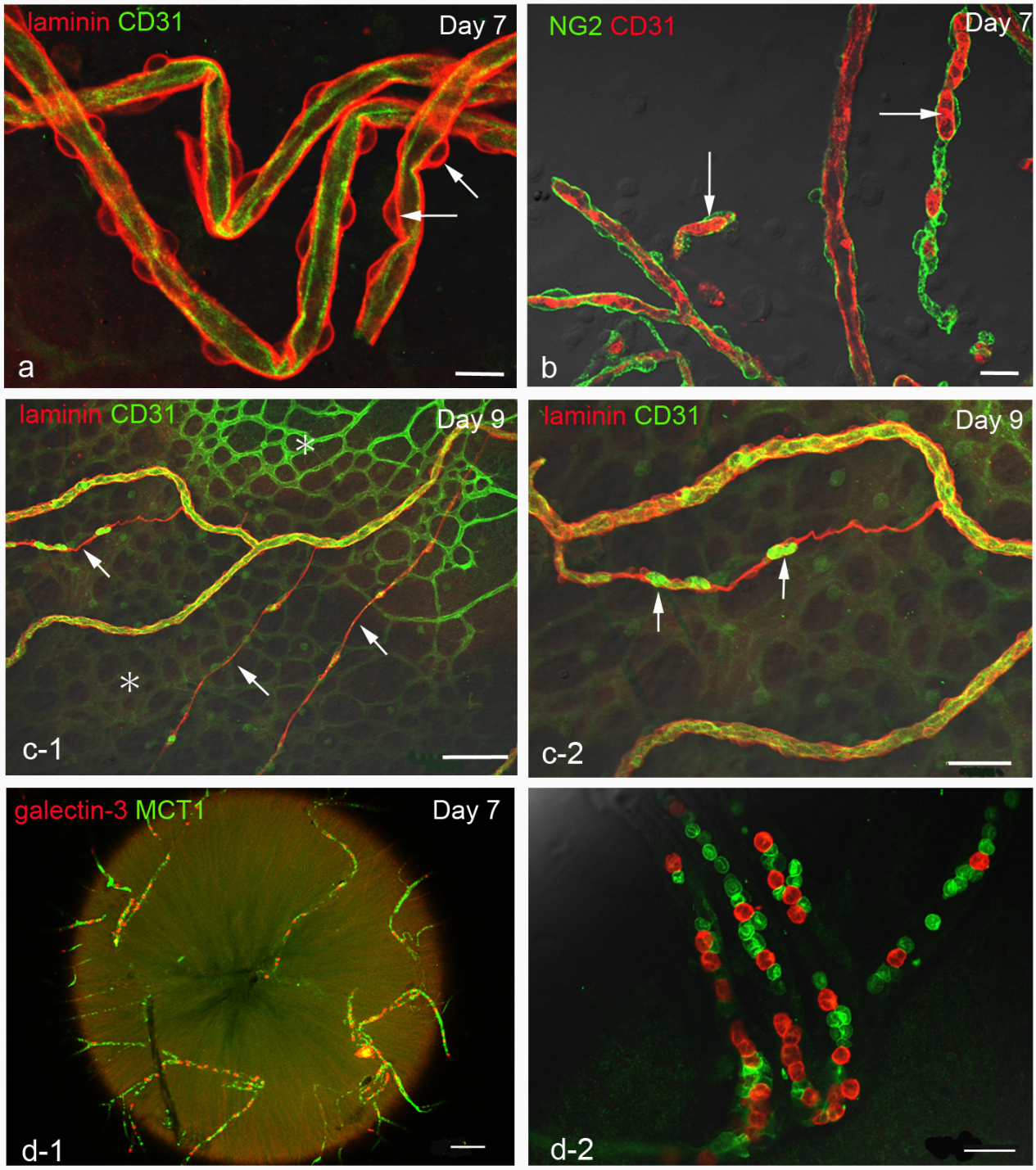
laminin CD31
Day 7
a
NG2 CD31
Day 7
b
laminin CD31
Day 9
c-1
laminin CD31
Day 9
c-2
galectin-3 MCT1
Day 7
d-1
d-2

Figure 6

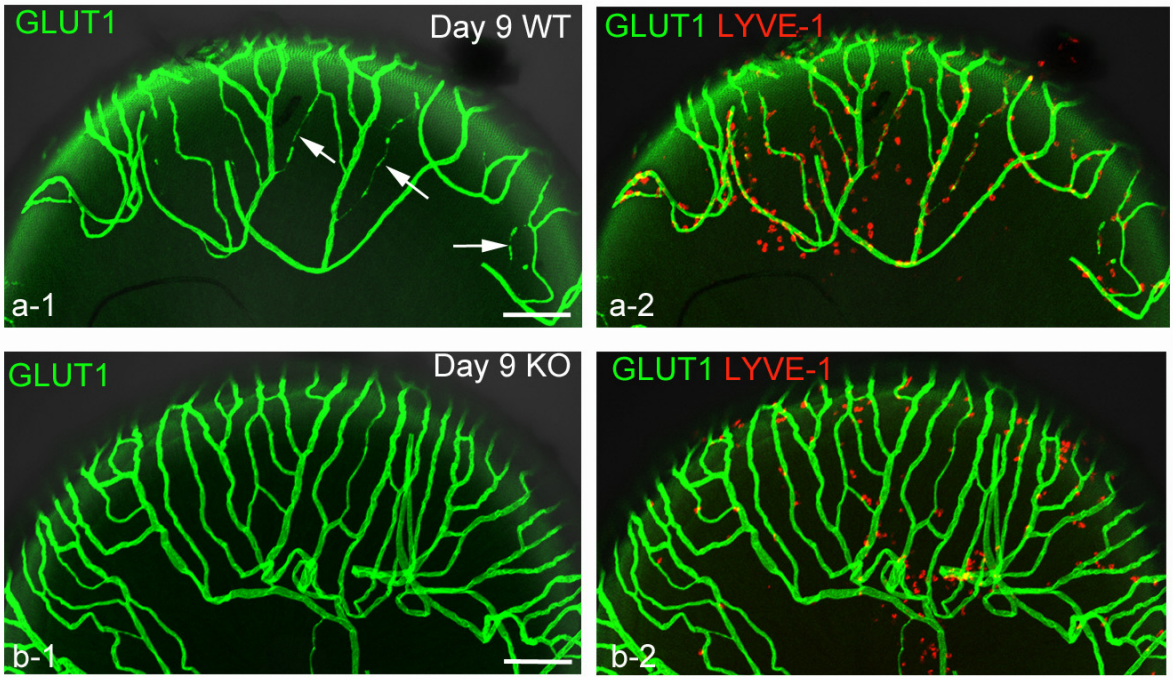
GLUT1
Day 9 WT
a-1
GLUT1 LYVE-1
a-2
GLUT1
Day 9 KO
b-1
GLUT1 LYVE-1
b-2

Figure 7