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# Bone marrow-derived microglia in pilocytic astrocytoma

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## 1. ABSTRACT

Tumour associated macrophages (TAMs) are increasingly recognized as supporters of tumour growth. The present study was undertaken to examine benign pilocytic astrocytomas (PAs) for the presence of M2 macrophages. We have asked the question whether TAMs in PAs share the predominant CD163 immunophenotype with tumour-associated microglia/macrophages of malignant gliomas. In addition, we were interested in the question whether there is evidence that the macrophages in PAs derive from resident microglia in surrounding normal brain or whether cells expressing a macrophage phenotype may invade PAs from the vasculature. The latter question is of great interest with regard to so-called "bone marrow-derived microglia" (BMDM) which may provide a physiological route of entry into the CNS that could be used for novel cell-based treatments of brain cancer. In fact, we have found strong morphological evidence for such macrophage recruitment into PAs. We propose therefore that PAs may be used as a model for the study of macrophage recruitment into gliomas. Importantly, our results also confirm that microglia/macrophage infiltration per se is not associated with malignant glioma behaviour.

## 2. INTRODUCTION

Brain tumours are the most common solid tumours in children, accounting for over 20% of cancers before age 15, and pilocytic astrocytomas (PAs) are among the most frequently occurring childhood brain tumours (1). PA is a relatively circumscribed, slowly growing, often cystic astrocytoma which shows benign biological behaviour. Its name derives from the "hair-like" (pilus, Latin for hair) appearance of tumour cells which possess very elongated cell processes. PAs are remarkably confined ("solid") when compared to astrocytomas of the diffuse type which invariably progress and become highly malignant. Thus, pilocytic and diffuse astrocytoma are prognostically very different tumour entities (2) and their differences in behaviour reflect distinct growth and tissue infiltration properties.

Microglia and brain macrophages are present in both PAs and diffuse astrocytomas (3) and even proliferate in PAs. In fact, Klein and Roggendorf (4) found the highest indices of proliferating microglia in astrocytomas in PAs with an average rate of 32% (+/- 6.8) of all proliferating cells. Microglia expressing MHC class II molecules are

**Table 1.** Basic clinical data of cases examined (juvenile PA WHO grade I)

| Case | Gender | Age | Location |
|---|---|---|---|
| 1 | M | 2 | Brain stem |
| 2 | M | 10 | Brain stem |
| 3 | M | 11 | Brain stem |
| 4 | F | 13 | Cerebellum |
| 5 | F | 5 | Cerebellum |
| 6 | M | 3 | Cerebellum |
| 7 | M | 7 | Cerebellum |
| 8 | F | 3 | Optic nerve L |
| 9 | F | 15 | Suprasellar |
| 10 | M | 9 | Suprasellar |
| 11 | M | 24 | Thalamus R |
| 12 | M | 7 | Thalamus L |

also present in PAs (5) but their number is lower than that of GLUT5-positive microglia (6).

CD163 is a haemoglobin scavenger receptor exclusively expressed in the monocyte-macrophage system (7). Specific antibodies directed against CD163 are now available which are suitable for routine use in paraffin-embedded tissue samples and offer an alternative to CD68 antibodies for identifying cells of monocyte/macrophage derivation (8). Recently, it has been shown that the number of microglia/macrophages with positive staining for CD163 and CD204, which are believed to be markers for M2 macrophages, is correlated with the histological grade of a glioma, and the ratio of M2 macrophages amongst tumour-associated microglia/macrophages was also associated with histological grade (9). The proportion of M2 microglia/macrophages and macrophage colony-stimulating factor (M-CSF) expression in tumour cells further correlated with the proliferative capacity of glioblastoma cells (9). PAs were not studied.

The immunosuppressive M2 macrophage phenotype is expressed by tumour-associated macrophages (TAMs), which constitute major infiltrates of solid tumours (10), and which contribute to tumour growth, invasion, and metastasis (11). TAMs represent the major inflammatory component of the stroma of many tumours, and can affect different aspects of the neoplastic tissue (12). With regard to M1 and M2 macrophage terminology, current thinking has replaced the "on/off " model of M activation with a more complex one in which macrophages exist in resting, classically, or alternatively activated forms (13). Classically activated macrophages are referred to as M1, and non-classically activated macrophages have been dubbed M2 (see ref. 13 for details).

The CD163 antigen is constitutively present in perivascular macrophages of the human CNS (14). They are the same perivascular cells that express the well-known ED2 molecule in rat CNS (15). Indeed, the ED2 antigen is the rat CD163 surface glycoprotein (16). CD163 is not a marker of normal ramified microglia and when seen on microglial cells may indicate a particular form of microglial activation (17). Alternatively, such cells may have invaded the CNS tissue from the bloodstream because ED2 has been shown to be expressed by bone-marrow derived microglia (BMDM) (18).

It was the purpose of this study to characterise a representative series of PAs with respect to their content of CD163 expressing (M2) macrophages. In addition, CD34 was used to assess the vascularisation of the tumour tissue investigated.

## 3. MATERIAL AND METHODS

### 3.1. Tissue samples

We selected tumour samples from patients with PAs operated at King Fahad Medical City (KFMC), Riyadh, KSA, between 2007 and 2009. All tumours were classified based on current WHO criteria (19). The 12 cases used in the study were selected by two neuropathologists (WAS, MBG) based on "typical appearance" in standard haematoxylin and eosin-stained paraffin sections. The study was reviewed and approved by the Institutional Review Board of KFMC. All cases were diagnosed as juvenile PAs WHO grade I. The average age of the patients was 9.1 years; the median age was 8 years. Basic clinical data are summarized in Table 1. Tumours from the most common locations of PAs such as brain stem, cerebellum, thalamus, suprasellar region and optic nerve were all represented.

### 3.2. Immunohistochemistry

Biopsies were fixed in 3.7% buffered formaldehyde (Avonchem) and embedded in paraffin. Immunolabelings for the two macrophage markers, CD163 and CD68, the CD34 stem cell antigen and the proliferation marker MIB-1 were performed on adjacent tissue sections using a Leica Bond-Max automated immunostainer system with diaminobenzidine (DAB) as the peroxidase substrate. The CD163 mouse monoclonal antibody recognises a membrane protein designated as M130 antigen, Ber-Mac3, Ki-M8 or SM4; it is present on all circulating monocytes and most tissue macrophages but not in those found in the mantle zone and germinal centres of lymphoid follicles, interdigitating reticulum cells and Langerhans cells (Novocastra, Leica Microsystems, product code: NCL-CD163). The monoclonal antibody directed against CD68 (Novocastra, Leica Microsystems, product code: NCL-L-CD68) also detects monocytes/macrophages but it highlights lysosomes giving the staining a more granular appearance. CD34 is a transmembrane protein expressed on immature haematopoietic stem/progenitor cells, capillary endothelial cells, embryonic fibroblasts and rare cells in nervous tissue (Dako, clone QBEnd-10, product code: M7165). Two cases were not labelled for CD34 due to limited tissue availability. The nuclear MIB-1/Ki-67 proliferation marker is preferentially expressed during all active phases of the cell cycle but is absent from resting cells. There appears to be no expression during DNA repair processes. MIB-1/Ki-67 was detected using a monoclonal antibody from Dako (product code: M7240). Sections were slightly counterstained with Mayer's haematoxylin. The two neuropathologists and YA evaluated all slides. All stains were performed using established laboratory and tissue controls for the antibodies employed.

YA and MBG also assigned numerical scores to the cases for each macrophage marker. A score of 0 was assigned if no immunoreactive cells were observed in a

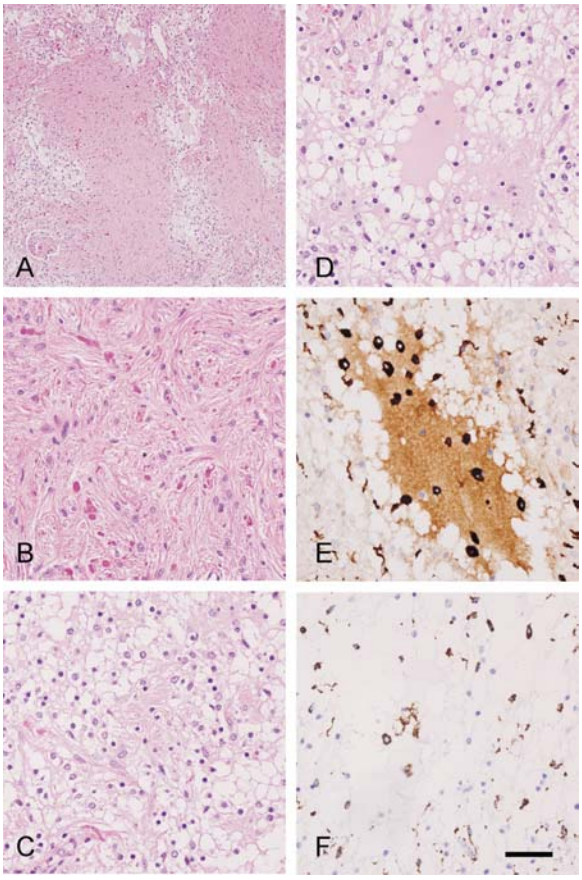


**Figure 1.** A-D Illustrate the biphasic pattern which is so typical of PA. There are varying proportions of compacted bipolar cells (B) associated with Rosenthal fibres (red, brightly eosinophilic structures in B) and loose-textured multipolar cells (C) associated with microcysts (D). Immunocytochemical labelling for the M2 macrophage antigen, CD163 reveals strongly immunoreactive rounded cells associated with a microcyst and strong diffuse staining of cyst content (E). CD68 immunostaining of an adjacent section labels fewer macrophage profiles within the microcyst and there is no diffuse labelling (F). Scale bar: 100 μm.

randomly chosen 40X microscopic field within the selected tumour area. A score of 1 was assigned if only a few immunoreactive cells were observed and a score of 2 was assigned if more than 10 immunoreactive cells were found while a score of 3 indicated a high number of immunoreactive profiles in a randomly chosen 40X microscopic field. The results demonstrated good interrater correspondence but due to the small number of cases from each region a statistical analysis was not performed.

### 3.3. *In silico* analysis

Biological associations representing functional interactions of CD163 were retrieved using PathwayStudio 6.1 software and the ResNet database of molecular interactions essentially as described previously (20). The microarray dataset used can be found at the National Centre for Biotechnology Information, Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo), GEO Series accession number GSE12657.

## 4. RESULTS

The classical biphasic pattern of PA is illustrated in Figures 1A-D. Figure 1A shows alternating compact and loosely textured tissue areas. Brightly eosinophilic Rosenthal fibres (19) which are typically found in fibre-rich tissue can be seen in Figure 1B. Figure 1C shows less dense tumour tissue and an associated microcyst in Figure 1D. Microcysts were strongly positive for CD163 demonstrating both diffusely immunoreactive material apparently containing CD163 in its soluble state (Figure 1E) and round cellular elements most likely representing macrophages which expressed high levels of CD163 (Figure 1E). A smaller number of microcyst-associated macrophages were CD68 immunoreactive (Figure 1F).

All PAs studied contained CD163 expressing macrophages (non-ramified cells) and usually even higher numbers of ramified, CD163 positive cells resembling microglia (Figure 2A). In the majority of cases, the number of CD163 immunoreactive cell profiles was higher than that of CD68 positive profiles (Figure 2B). Interestingly, it appeared that CD163 immunoreactivity was most pronounced and often even confined to tissue areas showing unequivocal tumour involvement. The only cells positive for CD163 in normal brain tissue (present in some biopsies) were perivascular cells (21).

Cellularity was higher in some tumours but the proliferation index was low in all and within the range for WHO grade I tumours (19). As expected, not all tumours studied showed Rosenthal fibres, and in a minority of cases the tissue pattern was compacted and uniform rather than biphasic. Within tumours showing a biphasic pattern, the vasculature appeared generally more prominent in the loose tissue areas.

As can be seen in Figures 2C and 2D, the presence of CD163 immunoreactive macrophages highlighted blood vessels because CD163 immunoreactive macrophages were more concentrated there. This illustrates a main finding of this study, the remarkable association of CD163 immunoreactive cells with blood vessel walls. CD163 immunoreactive cells appeared to transverse the latter, i.e. to leave the perivascular spaces and to migrate into tumour parenchyma. The area marked by the arrow in Figure 2C is shown at higher magnification in Figure 2D.

Blood vessels within neoplastic tissue areas were generally wider in diameter and more variable in size and shape compared with normal brain tissue. Blood vessels immunolabelled for CD34 can be seen in Figures 2E and 2F. CD34 immunoreactivity was not confined to blood vessels but occasionally also marked individual cells that could represent microglia/macrophages and/or their precursors (Figure 2F). However, all observers agreed that there was no obvious correlation between macrophage marker positivity and the presence of blood vessels in a given pilocytic tumour tissue area.

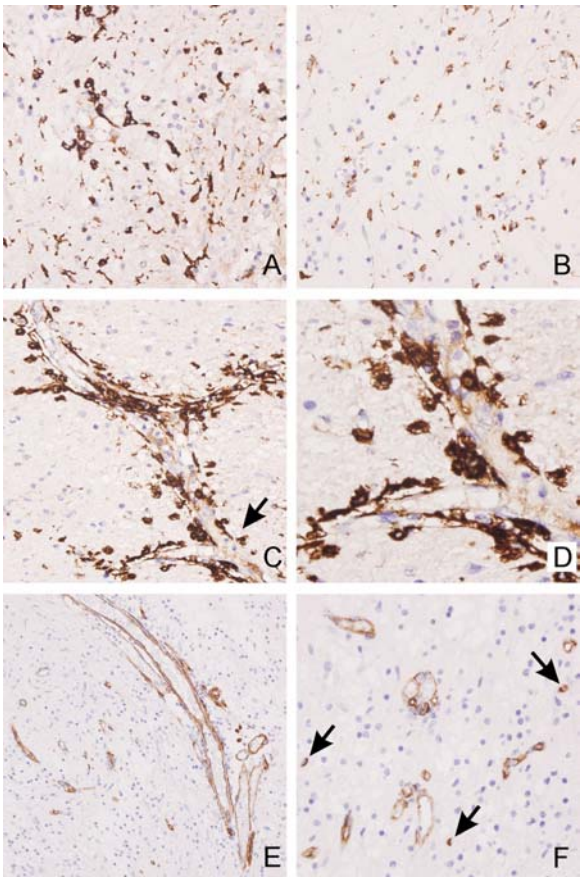


**Figure 2.** Ramified as well as round cells which express CD163 (A) and CD68 (B), respectively can be seen in association with both compact and loose-textured pilocytic tumour areas. The most striking finding of this study is the association of CD163 immunoreactive cells with blood vessel walls, which the former appear to cross from the bloodstream before invading the tumour (C, D). The area marked by the arrow in C is shown at higher magnification in D. E reveals larger, abnormal blood vessels staining for CD34 in a PA whereas F shows that CD34 immunoreactivity is also detectable in small round structures probably representing single cells (arrows). The photographs of both figures were taken from the same tumour.

Our *in silico* search for biological associations of CD163 revealed a number of interesting candidates for future histological studies, i.e. proteins interacting with CD163 whose expression is co-upregulated in PAs as indicated by the shades of red of the respective gene symbols shown in Figure 3: CSF-1, colony stimulating factor-1; HMOX1, heme oxygenase (decycling) 1; NR3C1, nuclear receptor, subfamily 3, group C, member 1 (glucocorticoid); ICAM1, intercellular adhesion molecule 1; LRP1, low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor).

## 5. DISCUSSION

Our observation that CD163 immunoreactive ramified microglia and, to a lesser extent, CD163 positive macrophages are present in significant numbers in PAs fits well with results of studies by other authors. Tanaka et al. (22) reported that on semiquantitative analysis, many Iba1-positive, microglia/macrophages were observed in PAs (19.9 +/- 6.5%). Iba1-positive, activated microglia/macrophages were commonly found in microcystic areas (22). The rather intense diffuse CD163 immunoreactivity observed by us in microcysts and also in compact tumour tissue of some cases may be explained by presence of the soluble form of CD163 (23), and it is tempting to speculate that CD163 could be useful as a biomarker for PAs.

In rectal cancer, expression of the macrophage antigen CD163 is associated with early local recurrence and reduced survival time (24). The same authors previously demonstrated that in breast cancer, expression of CD163 is associated with early distant recurrence and reduced patient survival (25). The authors consider CD163 expression by cancer cells following heterotypic fusion with TAMs a possibility. However, it is not clear how the highly up-regulated expression of CD163 in neoplastic lesions relates to the functions of this molecule. The human protein CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily (26). In vitro culture of human blood monocytes with recombinant M-CSF induces CD163 transcription (27). Heme, the functional group of haemoglobin, myoglobin and other haemoproteins, is a highly toxic substance when it occurs in the extracellular milieu (28) but it can be handled safely by two cellular systems, which both function by way of a circulating plasma carrier protein that upon ligand binding is recognized by a receptor. The haemoglobin-binding haptoglobin and the receptor CD163 represent one of them (28). Five different isoforms of CD163 have been described, which differ in the structure of their cytoplasmic domains and putative phosporylation sites (29). Haptoglobin preserves the CD163 haemoglobin scavenger pathway by shielding haemoglobin from peroxidative modification (30). Hp-Hb binding to CD163 is the primary mechanism of plasma Hb clearance, while clearance of Hb by direct binding to CD163 is secondary to Hp depletion (31).

The striking presence of numerous macrophages in association with microcystic areas in PAs as demonstrated in the present study may help to shed light on the development of PAs. PAs present as space occupying lesions resulting from both tumour growth and the development of a cystic component. The latter may become much larger than the actual tumour which then assumes the appearance of a mural nodule adherent to the inner cyst wall. Thus, it is tempting to speculate that inhibition of macrophage activity in PAs might be of therapeutic benefit: if cyst growth depends on macrophages, the space occupying effect of the tumour might be mitigated by interfering with macrophage activity. Microglia/macrophages in PA share the M2 (CD163) immunophenotype with tumour-associated microglia/macrophages of malignant glioma. However, as shown here and in line with previous observations on microglia/macrophages in PAs, macrophage infiltration of

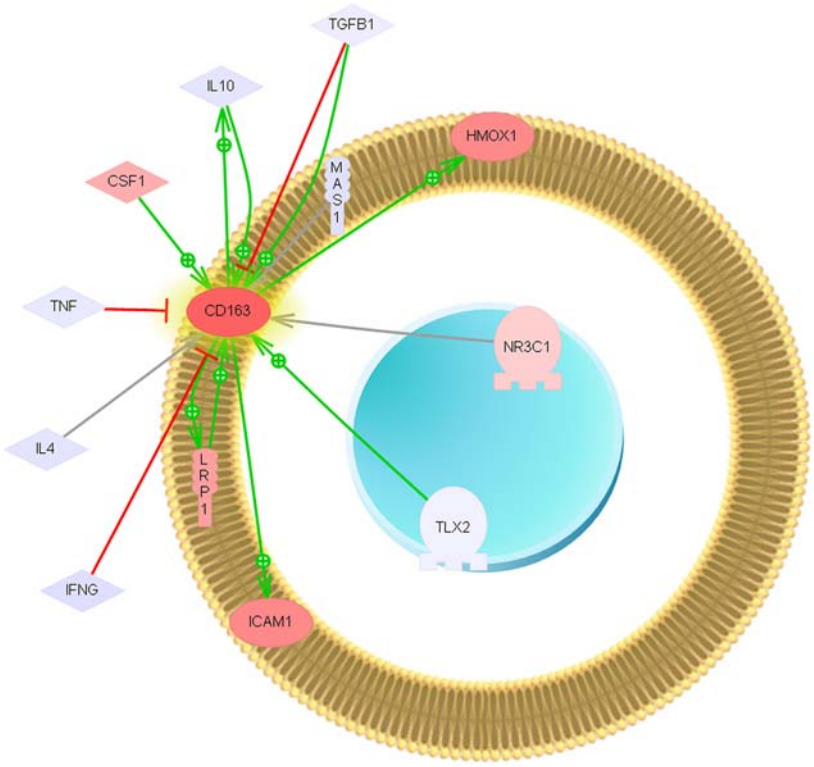


**Figure 3.** Biological interactions of CD163 in a schematised microglial cell/macrophage. The colours of the gene symbols indicate regulation by microglia/macrophages in PA vs. normal brain tissue. Proteins interacting with CD163 whose expression is co-upregulated are represented by red symbols. They represent candidates for future immunocytochemical studies. Display style for regulation: by effect; colour codes: green, positive regulation; red, negative regulation; grey, exact effect unknown. Abbreviations: CSF-1, colony stimulating factor-1; HMOX1, heme oxygenase (decycling) 1; ICAM1, intercellular adhesion molecule 1; IFNG, interferon gamma; IL-4, 10, interleukin-4, 10; LRP1, low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor); MAS1, MAS1 oncogene; NR3C1, nuclear receptor, subfamily 3, group C, member 1 (glucocorticoid); TGFB1, transforming growth factor, beta-1; TLX2, T-cell leukaemia homeobox 2; TNF, tumour necrosis factor; interactions between proteins are according to (25, 27, 56-71).

a glioma per se is not associated with malignant tumour behaviour.

The results of our study leave very little doubt that microglia/macrophages in PAs represent at least in part BMDM that have arrived via the vasculature (18). Therefore, PAs might be used as a model for the study of macrophage invasion in gliomas. Comparative systems biological (high-throughput) analyses of PA versus diffuse astrocytoma should prove useful in identifying the molecular characteristics that are key to malignant glioma growth. Serendipitously, BMDM trafficking suggests a physiological route of entry into the CNS that might be exploited for the development of novel cell-based glioma therapies (32, 33).

The finding that TAMs contribute to tumour growth, invasion, and metastasis has been demonstrated for a number of cancers including experimental malignant peritoneal mesothelioma (34). Therefore, a better understanding of the factors that govern macrophage infiltration of gliomas is very important. Recently, glypican-3 was identified as a molecule involved in the recruitment of M2-polarized TAMs in hepatocellular carcinoma (36). New technologies allowing the efficient genetic modification and ideally re-programming of TAMs are also needed. Zinc-finger nucleases (35) may represent one such a technology.

Many observations indicate that TAMs express several M2-associated pro-tumoral functions, including promotion of angiogenesis, matrix remodelling and suppression of adaptive immunity (37). M2-polarized TAMs in the invasive front of pancreatic cancer are associated with poor prognosis (38). Targeting TAM polarization may therefore become a complementary therapeutic strategy against cancer (39). Thus, detailed information on the mechanisms underlying M1/M2 macrophage polarization is of great interest.

Macrophages adapt their behaviour to signals coming from their microenvironment. While Th1 cytokines promote pro-inflammatory M1 macrophages, Th2 cytokines promote an "alternative" anti-inflammatory M2 macrophage phenotype (40). Consequently, TAMs are a central component of pathways connecting inflammation

and cancer (41). Macrophages can be differentiated to M1- or M2-type cells with either granulocyte macrophage colony-stimulating factor (GM-CSF) or M-CSF, respectively (42). M-CSF-driven monocyte-to-macrophage differentiation is associated with activation of cell cycle genes (43). Interestingly, IL-10 or IL-4 differentiate microglia towards a M2-deactivated or M2-alternatively-activated phenotype respectively (44), and during M-CSF-mediated differentiation of monocytes into macrophages under conditions that promote an M2 alternatively activated macrophage phenotype, the expression of Sema3A receptors (neuropilin-1 (NRP-1), NRP-2, plexin A1, plexin A2, and plexin A3) increases significantly (45). Toll-like receptor (TLR) 2, 4, 7, and 9 agonists, together with adenosine A2A receptor (A2AR) agonists, switch macrophages from an inflammatory (M1) to an angiogenic (M2-like) phenotype (46). Similarly, PPARgamma activation skews human monocytes toward an anti-inflammatory M2 phenotype (47). However, only native monocytes can be primed by PPARgamma activation to an enhanced M2 phenotype (47). PPAR agonists are potentially useful for inhibiting the early phases of tumorigenesis in inflammation-driven cancers through their antagonistic effect on M1. In more established tumours, the macrophage phenotype is more diverse, making it more difficult to predict the outcome of PPAR agonism (48).

Taken together, microglia/macrophages and glioma cells appear to have an intricate and symbiotic relationship (49-54). The results of the present study suggest that this also holds true for PAs. In addition, bone marrow-derived microglia exhibiting an M2 macrophage phenotype are suggested to enter PAs via the blood stream. Whether tenascin plays a role in regulating this cell trafficking remains to be determined (55).

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