

# Expression of CD34 and CD146 vascular markers contributes to the immunological function of the human palatine tonsil

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**Summary.** The fundamental function of the palatine tonsil is the immune response to airborne and foodborne pathogenic agents. Small blood vessels have an important role in the provision of a special micro-environment in which the immune response occurs. In this study, we investigated the expression of vascular markers CD34 and CD146 and basal lamina marker - type IV collagen - in the small blood vessels of the human palatine tonsil in the context of their role in the immunological function of the tonsil. The tonsils were collected after tonsillectomy from ten patients with chronic tonsillitis, aged 18-28 years. Five- $\mu$ m-thick paraffin sections were routinely stained with haematoxylin and eosin, while the studied markers (CD34, CD146 and type IV collagen) were detected immunohistochemically using LSAB2/HRP method. CD34 was expressed equally in the capillaries within and below the crypt epithelium, in lymphoid follicles and in high endothelial venules localized para- and interfollicularly. CD146 molecule was expressed on the luminal surface of endothelial cells in the capillaries of the crypt epithelium, while its expression in high endothelial venules was seen on the luminal and lateral surfaces of the cuboidal endothelial cells. In contrast to the basal lamina of intraepithelial capillaries, where collagen IV-immunopositivity is mostly seen as a continuing line, the basal lamina of high endothelial venules was seen as a two- or three-layered structure

beneath the cuboidal endothelial cells. The specifics of expression of CD34, CD146, and type IV collagen confirm the morphofunctional specialization of endothelium in crypt epithelium capillaries, and also in endothelium of high endothelial venules, which is directly associated with the role of these vessels in the immune function of the tonsil.

**Key words:** Human palatine tonsil, Microvasculature, Endothelium, Immunohistochemistry

## Introduction

As a part of the Waldeyer's ring, palatine tonsils represent the first line of defence against airborne and foodborne pathogens and are responsible for the mucosal and systemic immunity. The initial stage of an immune response occurs in the crypt epithelium which has a role in antigen capturing and processing to immunocompetent T and B cells (Maeda and Mogi, 1984; Perry, 1994), responsible for the cellular- and humoral-mediated immune response in the tonsillar lymphoid tissue (Brandtzaeg and Halstensen, 1992; Perry and Whyte, 1998; Nave et al., 2001; Avramović et al., 2011). In the provision of a specific microenvironment in which the immune response occurs, capillaries and high endothelial venules (HEVs) have an important role (Akkus et al., 2004; Hafeez et al., 2008; Jović et al., 2015).

CD34 molecule is the principal marker of endothelial cells in embryonal, fetal, and adult blood vessels. It is a transmembrane protein from the

sialomucin family, which by way of binding to L-selectin on leukocytes enables their adhesion to the surface of vascular endothelial cells, supporting thus the passage of leukocytes through the blood vessel wall (Fina et al., 1990; Krause et al., 1996; Satoma et al., 2002). Vascular endothelial cells also express CD146 molecule (MelCAM, MUC18/S-Endo), a transmembrane adhesion glycoprotein from the superfamily of immunoglobulins. In addition to vascular endothelial cells, its expression has been confirmed on malignant melanoma cells, smooth muscle cells, and follicular dendritic cells (Bardin et al., 2001). Recent studies have suggested a role of CD146 in numerous physiological and pathological processes, such as cell migration and proliferation, cell signaling during embryonal development and organogenesis, initiation of angiogenesis in tumor tissues and immunological processes (Wang and Yan, 2013).

Basal lamina of all the epithelia in the human body, including the vascular endothelium, contains an adhesion glycoprotein, type IV collagen, which together with laminin, nidogen-1 and -2, and perlecan - proteoglycan, forms a three-dimensional molecular structure as a mechanical scaffold for epithelial cells and as a selective barrier (Otsuki et al., 1990; Kühn, 1995; Pöschl et al., 2004).

Considering the fact that there is no consensus among researchers as to the distribution of small blood vessels in the human palatine tonsil, our aim in this paper was to examine the expression of vascular markers CD34 and CD146 and a basal lamina marker, type IV collagen, in the small blood vessels of the human palatine tonsil in the context of their role in the immunological function of the tonsil.

## Material and methods

Palatine tonsils were obtained from the Clinic of Otorhinolaryngology, Clinical Center Niš, and were taken from ten patients, aged 18-28 years, who had undergone elective tonsillectomy due to chronic tonsillitis. Prior to tonsillectomy, patients' written consent was obtained, as well as the approval from the Ethical Committee of the Clinical Center Niš (No. 9060/14) for the study.

Tissue samples were routinely processed all the way to paraffin blocks. Five- $\mu$ m-thick sections were stained with hematoxylin and eosin (H&E) for routine histological analysis and immunohistochemically using the LSAB2/HRP method to mark the endothelium and basal lamina of the blood vessels. In brief, immunohistochemistry involved deparaffinization of the sections in xylol, washing in gradually decreasing alcohol concentrations and in distilled water. Antigen retrieval was done by incubating the sections for 90 minutes in trypsin; endogenous peroxidase blocking was done in 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes; after that, incubation at 4°C over night was performed, with the following primary monoclonal antibodies: (1) antibody for CD34 molecule

(M716501, Dako, dilution 1:50) and antibody for CD146 molecule (Ab49492, Abcam, dilution 1:100), for endothelial cell marking, and (2) antibody to collagen IV (Ab6586, Abcam, dilution 1:100) to mark the basal lamina of blood vessels; all the dilutions of primary antibodies were prepared with the Dako antibody diluent (Code S0809, Dako North America, USA). After the sections were incubated in LSAB2 System-HRP (Code K0673, Dako, USA) for 30 minutes, they were stained with chromogen DAB (3,3'-diaminobenzidine tetrahydrochloride, Code K3468, Dako), in a controlled fashion under the microscope until the appearance of a positive immune reaction. After dehydration with increasing alcohol concentrations, contrast enhancement with Mayer's hematoxylin and after being made translucent in xylol, the sections were mounted in Canada balsam.

Microphotographs of the tissue sections were taken using the Leica DFC295 (Leica Micro-Systems, Reuil-Malmaison, France) digital camera on a light microscope Olympus BX-50.

## Results

The undertaken histological analysis revealed a change in the architecture of the crypt epithelium due to expressed infiltration of lymphocytes, so that the structure of the stratified squamous epithelium could be noticed only in certain parts and owing to the presence of one to two layers of squamous cells on the surface of the epithelium; a borderline between epithelium and subepithelial lymphoid tissue is unclear (Fig. 1A). Small blood vessels that morphologically resembled capillaries are present in the crypt epithelium (Fig. 1A). In the interfollicular lymphoid tissue, HEVs are seen with large cuboidal endothelial cells containing a centrally positioned nucleus and abundant bright cytoplasm (Fig. 1B).

A CD34-immunopositive reaction was seen in the crypt epithelium (Fig. 2A,B) and parafollicularly in the lymphoid tissue of the tonsil (Fig. 2C,D). In the crypt epithelium, CD34 immunopositivity was present only on capillary endothelial cells. The greatest density of immunopositive capillaries was observed on the borderline between the crypt epithelium and mantle zone of the lymphoid follicle located subepithelially (Fig. 2A). Granulocytes, recognisable for their segmented nucleus, adhere to the apical surface of CD34-immunopositive endothelial cells, whereas lymphocytes closely attach to the basal surface of capillary endothelial cells (Fig. 2B). Endothelial cells of the HEVs were also immunopositive (Fig. 2C), with the greatest intensity of reaction on the apical surface of the cuboidal endothelial cells, which are not of the same size and shape, especially at the site of lymphocyte passage through the wall of the HEVs (Fig. 2D).

A CD146-immunopositive reaction in the structurally changed and lymphocyte-infiltrated crypt epithelium is located in the capillaries and is manifested

## CD34 and CD146 in human palatine tonsil

as a continuing brown line on the luminal side of the endothelium; in some capillaries, a discontinuity of immunopositive reaction was noticed, especially at the sites where small groups of lymphocytes were visible on the capillary wall (Fig. 2E). Endothelial cells of the HEVs demonstrated a CD146-immunopositive reaction of variable intensity on their luminal and lateral surfaces (Fig. 2F).

In all morphological compartments of the tonsil, blood vessels were immunopositive to type IV collagen (Fig. 3). Immunopositivity of the capillary basal lamina in the crypt epithelium (Fig. 3A-C) was mostly seen as a continuing line, which was in some capillaries discontinued at the sites where small groups of lymphocytes were gathered to the capillary wall (Fig. 3C). Type IV collagen-immunopositive reaction revealed a large number of very tiny capillaries in the lymphoid follicles, whereas immunopositivity localized parafollicularly corresponds to HEVs (Fig. 3D). Type IV collagen immunopositive basal lamina in HEVs (Fig. 3E,F) had often the appearance of a two- or three-layered structure beneath the cuboidal endothelial cells (Fig. 3F).

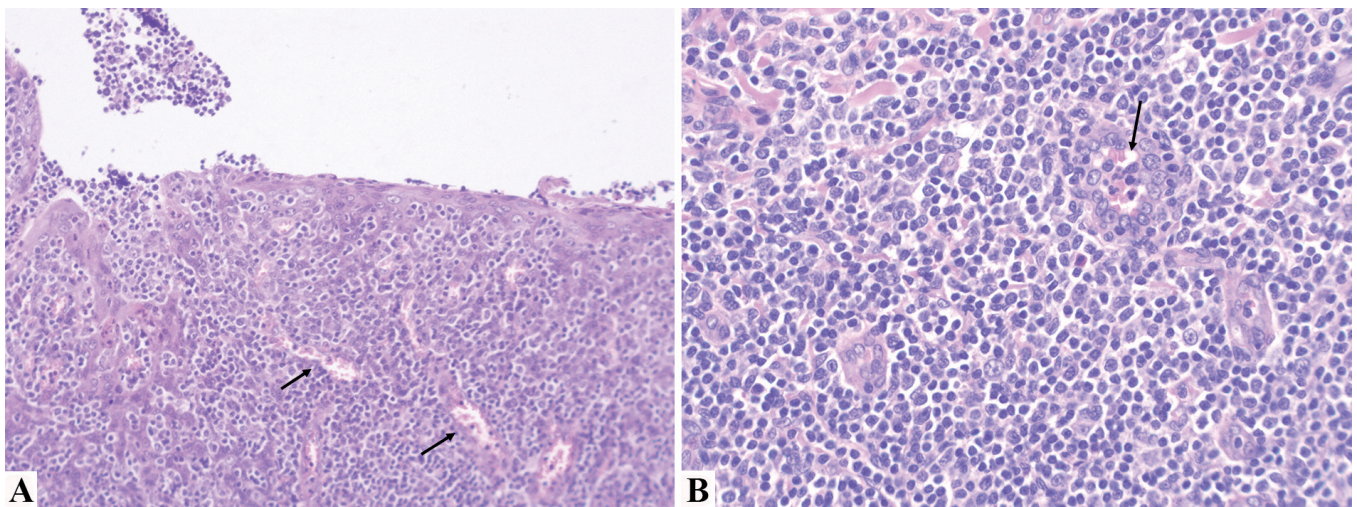
### Discussion

With the intention to associate blood vessel localization with their role in the immunological function of the tonsil, we tried to present the distribution of small blood vessels in the human palatine tonsil using the anti-CD34, anti-CD146, and anti-type IV collagen monoclonal antibodies.

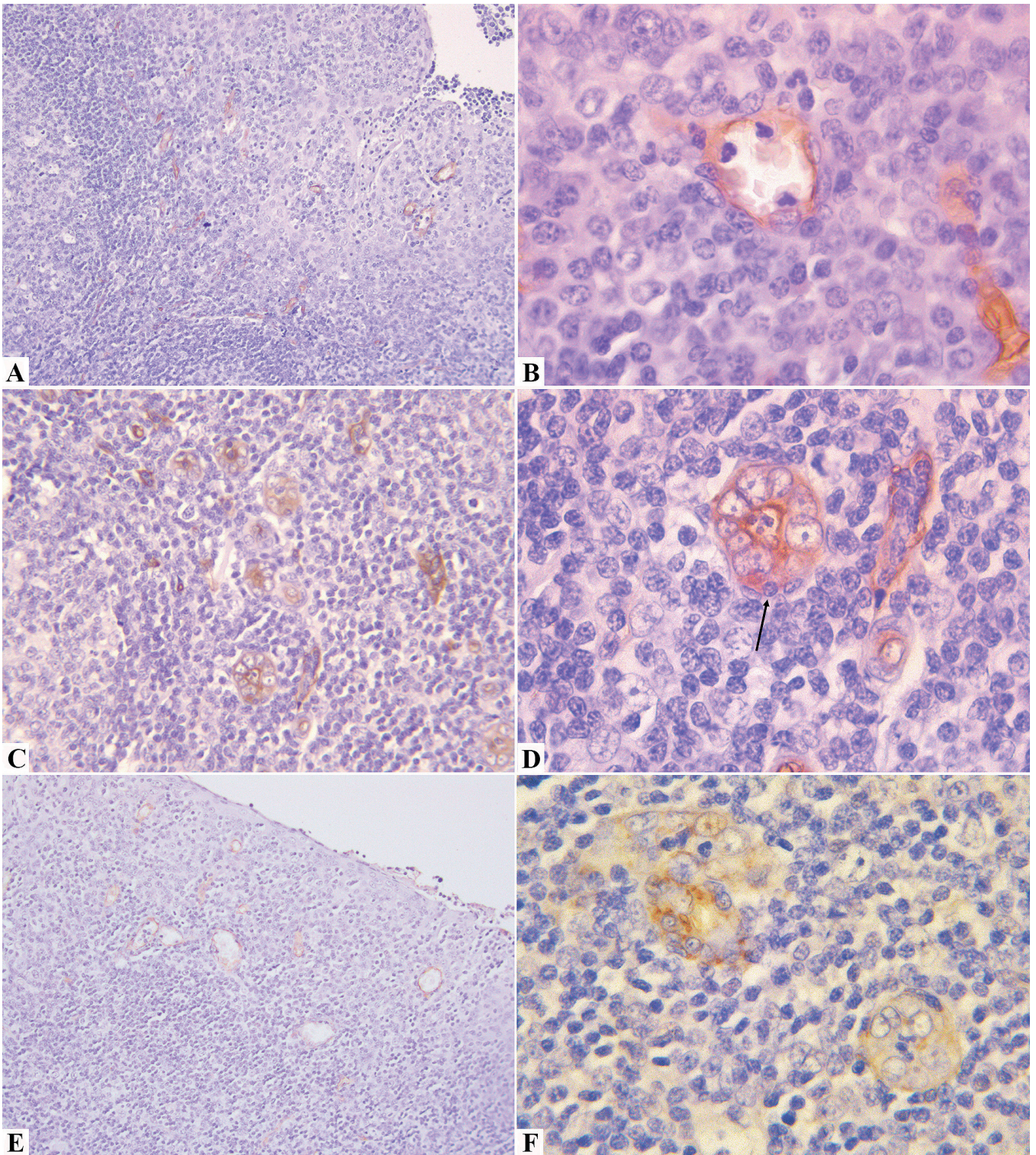
Similar to other anatomical localizations in the body, tonsillar capillaries have a role in the exchange of

nutrients between the blood and tissues, while HEVs enable colonization of lymphoid tissue with lymphocytes and lymphocyte homing (Perry et al., 1992a; Perry and Whyte, 1998; Akkus et al., 2004). In the above processes, small blood vessel endothelium represents a mechanical barrier between the blood and tissue, and depending on the tissue microenvironment, it shows the specifics related to intercellular junctions, cell adhesive molecules, cytokine production, and basal lamina properties. Expression of cell membrane proteins, termed vascular markers, is common for all endothelia; CD34 and CD146 being among them.

By way of histological analysis and based on the expression of vascular markers and type IV collagen, the presence of capillaries was confirmed in different morphological compartments of the palatine tonsil, including the crypt epithelium. It is well known that epithelium is an avascular structure, except for the *striae vascularis* in the inner ear, so that the presence of capillaries in the crypt epithelium, morphogenetically determined as some authors believe (Schmedtje and Batts, 1973; Favre et al., 1986), makes the tonsil specific compared to other organs. Schmedtje and Batts (1973) defined those capillaries as sinusoids, explaining them as the sites where intraepithelial immunoglobulin-producing cells secrete immunoglobulins, while Brandtzaeg (1984) thought that the capillaries serve to supply the crypt epithelium with serum immunoglobulins, supporting thus mucosal immunity. Perry et al. (1992a), based on their research of microvasculature of the human palatine tonsil, proposed that capillaries in the crypt epithelium, in addition to subepithelial lymphocyte invasion, could be considered a supplemental source of lymphocytes that infiltrate the

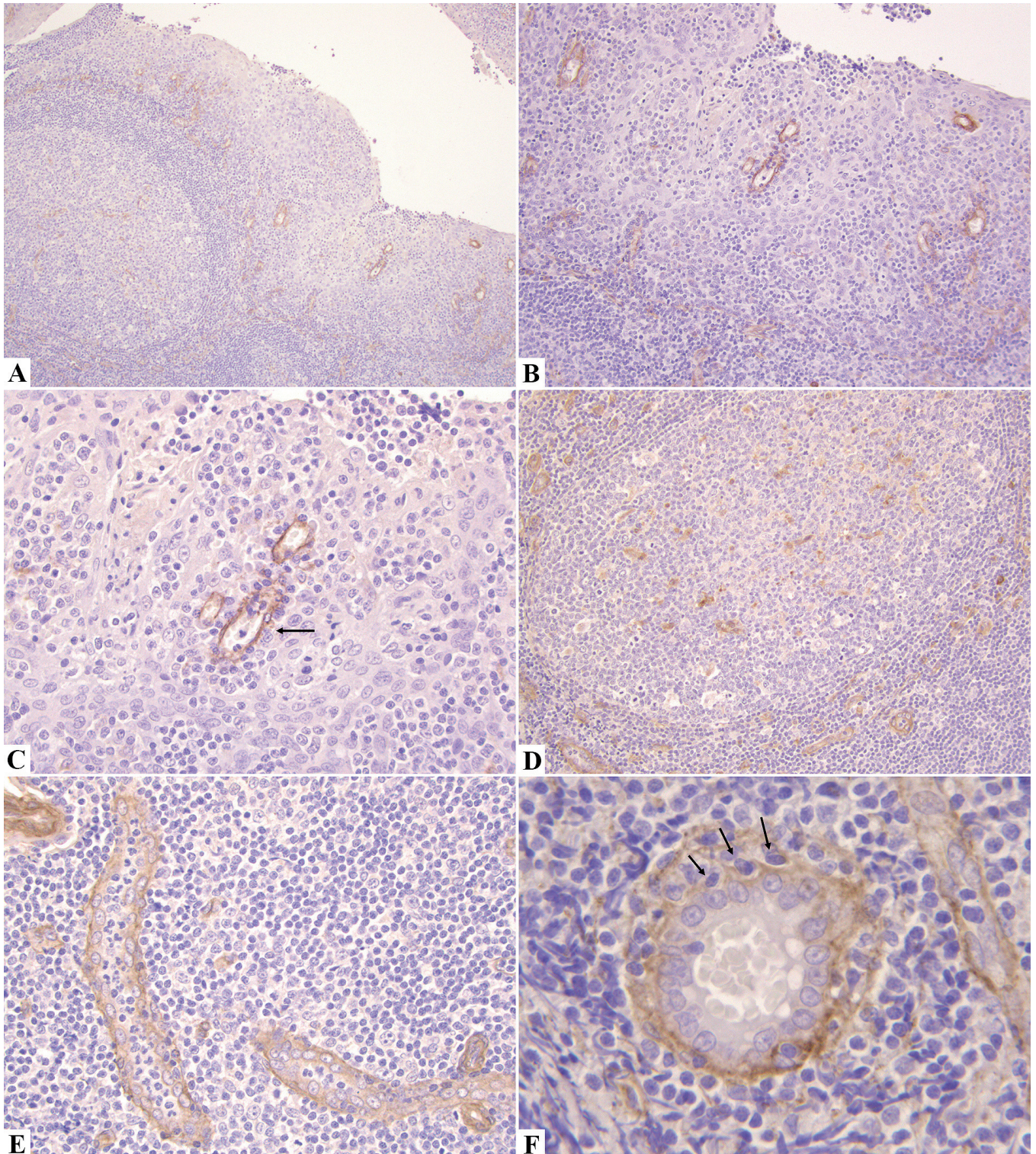


**Fig. 1.** H&E stained palatine tonsil. **A.** Crypt epithelium is strongly infiltrated with lymphocytes; sporadic islands - bands of stratified squamous epithelium can be noticed; in the epithelium, small blood vessels morphologically resembling capillaries (arrows) can be seen, with red colored erythrocytes in the lumen. **B.** HEVs in tonsillar interfollicular region; erythrocytes and lymphocytes (arrow) can be seen in the lumen of the HEV. A, x 200; B, x 400.

*CD34 and CD146 in human palatine tonsil*

**Fig. 2.** Expression of CD34 (**A-D**) and CD146 (**E and F**) molecules in the palatine tonsil - LSAB2/HRP method. **A, B.** Crypt epithelium, containing the capillaries with immunopositive endothelial cells. **B.** Granulocytes adhering to the apical surface of endothelial cells can be seen in the capillary lumen. **C.** Parafollicularly localised HEVs with CD34-immunopositive cuboidal endothelial cells. **D.** A detail from the preceding figure showing a lymphocyte (arrow) in its stage of passage through the HEV wall. **E.** Capillaries in the crypt epithelium showing CD146-immunopositivity in the form of a continuing brown line on the luminal surface of the endothelium; in some capillaries, a line-like immunopositive reaction is not of equal intensity along the entire wall circumference, and in some capillaries the reaction is absent. **F.** HEVs - in the figure on the left, a reaction of CD146-immunopositivity of variable intensity can be seen on the luminal and, sporadically, on the lateral surfaces of the cuboidal endothelial cells. A, E, x 200; B, D, x 1000; C, x 400; F, x 800.

## CD34 and CD146 in human palatine tonsil



**Fig. 3.** Type IV collagen expression in the palatine tonsil - LSAB2/HRP method. **A-C.** In the crypt epithelium, immunopositive capillary basal laminas can be seen. **C.** A detail from the previous figure: the basal lamina is interrupted on the site of lymphocyte passage through the capillary wall (arrow). **D.** Para- and intrafollicular blood vessels with collagen IV expression. **E, F.** HEVs showing a strong collagen IV-immunopositive reaction in longitudinal (**E**) and transversal (**F**) sections. **F.** Basal lamina looking as a three-layered structure on the site where lymphocytes are present (arrows) in their phase of migration through the HEV wall; one lymphocyte adheres to the luminal surface of the cuboidal endothelial cell. A, x 100; B, D, x 200; C, E, x 400; F, x 800.

crypt epithelium. Our results confirmed the earlier interpretation of the role of intraepithelial capillaries. In particular, in a recently published electron microscopic study of the human palatine tonsil, we have shown fenestrated capillaries in the crypt epithelium and subepithelially (Jović et al., 2015), and in this paper, on the basis of type IV collagen expression, we demonstrated a discontinuity of capillary basal lamina on the site of lymphocyte grouping, indicating a lymphocyte passage through the capillary wall.

A specific pattern of expression of adhesion molecules in the crypt epithelium, i.e. CD34-immunopositivity on the luminal surface of endothelial cells and discontinuity of a line-like immunopositive reaction on CD146 and collagen type IV, points to the role of capillaries in transendothelial migration and population of the crypt epithelium by leukocytes, mostly lymphocytes. Maeda and Mogi (1984) found that lymphocytes in the crypt epithelium populate intraepithelial canals - microcrypts, which on the surface of the crypt epithelium open as pores, containing a "plug" of M-cells, whose role is to capture and process antigens from the crypt lumen to the lymphocytes, which further participate in the local immune response.

Bearing in mind the fact that lymphocyte population in the crypt epithelium and interfollicular regions is mostly composed of CD4<sup>+</sup> cells that include both naïve (CD45RA<sup>+</sup>) and memory (CD45RO<sup>+</sup>) subsets (Brandtzaeg, 2003), as well as our finding of the multilayered and fragmented basal lamina of the HEV, we can suppose that HEVs in interfollicular regions are the major source through which CD4<sup>+</sup> cells populate T-dependent tonsil areas, from which they further migrate into the crypt epithelium, joining the intraepithelial lymphocytes that have left the circulation at the level of capillaries.

Earlier studies have shown CD146 expression, in addition to the luminal surface, in the region of intercellular junctions as well, based on which it has been proposed that CD146 plays a role in the preservation of endothelial integrity, i.e. in the control of intercellular cohesion, paracellular permeability, and leukocyte transmigration (Bardin et al., 2001; Jouve et al., 2013). In this study, a different manner and intensity of CD146 expression was observed in the crypt epithelium capillaries and in HEVs, suggesting the morphofunctional specifics of endothelial cells in these blood vessels, especially in HEVs. Studies in the past have reported the expression of leukocyte antigen-1 (LFA-1) and intercellular adhesion molecule (ICAM-1), attributed with a role in transendothelial migration of lymphocytes through the HEV wall (Perry et al., 1992b). More recently, a nuclear factor preferentially expressed on the cuboidal endothelial cells of HEVs has been defined, termed nuclear factor from HEV (NF-HEV), supposed to have a key role in the control of formation of a specialized phenotype of HEV endothelial cells (Baekkevold et al., 2003). One of the specifics of HEV endothelial cells is LYVE-1 (lymphatic endothelium

hyaluronan receptor) expression, present exclusively on the lymph vessel endothelial cells, but not on the endothelial cells of blood capillaries in adults (Wrobel et al., 2005). In addition to this, two endothelial adhesive molecules have been defined, termed vascular adhesion protein-1 (VAP-1) and peripheral node addressin (PNAd), representing a set of sialomucins on HEV endothelial cells binding L-selectin on the lymphocyte membrane (Akkus et al., 2004; Uchimura and Rosen, 2006). By way of interaction with its ligand, CD34 molecule has a pro-adhesive role and helps lymphocyte homing (Furnes and McNagny, 2006), as confirmed by our finding of lymphocytes adhering to the luminal surface of HEV endothelial cells. Studies have shown that HEV endothelial cells have a number of membrane glycoproteins which can be modified by specific glycosylation, and that modified CD34 form, expressed only on the cell membrane of cuboidal endothelial cells of HEVs, serves exclusively to bind L-selectin (Furnes and McNagny, 2006).

Proteoglycans with glycosaminoglycans represent the main extracellular matrix molecules. The only representative of proteoglycans in the basal lamina is the proteoglycan heparan sulfate - perlekan. The content of HEV basal lamina deviates from the generally accepted composition of basal lamina constituents, since it has been confirmed that, in addition to the proteoglycan heparan sulfate, the basal lamina of HEVs contains the proteoglycan hondroitin sulfate, suggesting a potential role of the molecule in supporting lymphocyte migration after their passage through the layer of cuboidal endothelial cells of HEVs (Sunami-Kataoka et al., 2001). In our study, the appearance of the HEV basal lamina as a three-layered collagen IV-immunopositive structure in the areas of lymphocyte grouping, clearly suggests the role of the basal lamina and surrounding extracellular matrix in the passage of lymphocytes through the HEV walls. Our findings have been corroborated by the recently published data according to which collagen IV, binding to CCL21, CXCL13 and CXCL12 lymphoid chemokines, forms a microenvironment in which chemokines support a direct passage of lymphocytes through the HEV wall. In that way, helped by type IV collagen, the basal lamina of HEVs assumes the role of a guide in leukocyte migration (Yang et al., 2007). With regard to the role of "local" extracellular matrix in the transport of lymphocytes through the HEVs, Usui et al. (2002) demonstrated the expression of mac25/angiomodulin on inflammation-activated endothelial cells of the HEVs, putting it in connection with the role of mac25/angiomodulin in linking humoral factors produced in the vicinity of HEVs.

In our study, the localization of capillaries and HEVs in the lymphoid tissue matches the results of light microscopy and scanning electron microscopy analysis of blood vascular architecture of the musk shrew tonsil (Doi et al., 2003), i.e. there is a network of capillaries subepithelially in the lymphoid tissue, and HEVs are localized more parafollicularly and less interfollicularly.

## CD34 and CD146 in human palatine tonsil

The mean number of HEVs in the human palatine tonsil reveals that HEVs are most dense immediately beneath the region of intense reticulation of the crypt epithelium, which is attributed to the degree of antigen stimulation of the tonsil (Hafez et al., 2008). Unlike the findings of Hafez et al., our study has demonstrated that the capillaries, but not the HEVs, are the most numerous between reticular crypt epithelium and mantle zone of the lymphoid follicles located subepithelially; however, we agree with the authors that the density of the microvasculature is related with the degree of antigen stimulation of the tonsil.

Based on our results, corroborated with the results of other researchers, we consider that CD34 molecule expressed on the cuboidal cells of HEVs has a proadhesive role, while CD146, expressed on both the luminal and lateral surfaces of endothelial cells, initiates cellular signals which at the level of intercellular junctions regulates the passage of lymphocytes. The expression of CD34 and CD146 molecules facilitates T and B cells extravasation into T- and B-dependent zone of the tonsil, as well as the formation of a specific extracellular matrix with proteoglycans and various humoral factors. If we add here our results of the expression of CD34 and CD146 in the capillaries of the crypt epithelium, then it could be concluded that the examined markers indirectly enable humoral and cellular immune response of the tonsil (Bykova and Stadykova, 2002; Brandtzaeg, 2003).

In conclusion, the observed specific pattern of expression of vascular markers CD34, CD146, and type IV collagen suggests the role of intraepithelial capillaries and HEVs in the transendothelial passage of lymphocytes and population of the tonsil morphological compartments, providing thus the conditions for the immunological function of the tonsil.

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*CD34 and CD146 in human palatine tonsil*

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