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Extracellular matrix proteins and integrin receptors in reactive and non-reactive lymph nodes

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SUMMARY

The extracellular matrix (ECM) proteins collagen I, III and IV, laminin, fibronectin, vitronectin, thrombospondin, tenascin and their integrin receptors of the β_1 and β_3 subfamilies showed characteristic patterns of distribution in different compartments of non-reactive and reactive lymph nodes (human and monkey). This was particularly evident during development of germinal centres. Thus, ECM proteins (collagens, laminin, fibronectin and tenascin) were abundant in the interfollicular (T-cell rich) compartments of non-reactive as well as reactive lymph nodes. In primary follicles, collagen I, III and fibronectin were expressed but displaced by the expanding germinal centre during the formation of secondary follicles in reactive lymphoid tissues. The integrin subunits were mainly associated with endothelial cells and lymphoid cells in interfollicular areas, but were absent or only poorly expressed in primary as well as secondary follicles. Evidently the expression of ECM components and their integrin receptors is markedly down-regulated in the reactive, highly proliferative germinal centres.

INTRODUCTION

The extracellular matrix (ECM) triggers instructive signals at the cell surface through ECM–ligand/integrin interactions, which after transduction can regulate gene expression leading to cell and tissue changes related to characteristic morphogenesis and/or cell function.[1]

Cells in lymphoid tissues, as well as other tissues, are surrounded by a fine web of ECM mainly composed of collagens, fibronectin, laminin and vitronectin, which determines the particular tissue microenvironment and its functional characteristics. Stages of immune responses are reflected by histological changes in the various compartments of the lymph node, i.e. the follicles, medullary cords, paracortex and sinuses.[2] Thus, humoral responses are characterized by a marked metamorphosis of primary follicles to secondary follicles, which has been studied extensively with regard to differentiation, interaction and proliferation of various cells components. However, ECM and their receptors are poorly characterized in relation to defined functional states of lymph nodes. Some receptors involved in the binding to various ECM proteins have recently been studied in lymphoid tissues. Thus, the presence of receptors belonging to the integrin family of adhesion molecules was demonstrated, including the β_1 [CD29/CD49a-CD49f or very-late activation antigen (VLA) proteins] and β_3 (CD61/CD41 and CD51 or cytoadhesins) subfamilies, which bind to fibronectin, laminin, vitronectin and collagen.[3]

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The expression of some integrins and ECM proteins in reactive lymphoid tissues has also been reported recently[4,5] but little information is available on non-reactive lymph nodes. In the present study we have compared the expression of the most widely expressed ECM proteins and their integrin receptors on non-reactive and reactive lymphoid tissues. For reasons of availability, both human and cynomolgus monkey specimens were examined and compared as an extension of previous studies.[6]

Our observations indicate clear differences in the presence of ECM proteins and their integrin receptors in the different lymph node compartments, and a redistribution during reactive germinal centre formation.

MATERIALS AND METHODS

Specimens

Hyperplastic tonsils ($n = 6$) from children and reactive lymph nodes taken during abdominal surgery were processed within 15–20 min after surgery. Non-reactive and hyperplastic cervical lymph nodes from healthy cynomolgus monkeys ($n = 7$) were taken and processed as the human material, as described previously.[7] All biopsies were partly snap frozen in liquid nitrogen, and stored at $-70°$, and partly fixed in 4% paraformaldehyde for conventional histology. Eight-micrometre thick frozen sections were prepared and fixed in acetone for 10 min, and stored at $-20°$ until use.

Immunohistochemistry

Frozen sections of human and monkey origin were blocked for endogenous peroxidase activity with hydrogen peroxide in

Table 1. Antibodies to ECM proteins and their receptors

Reactivity	Antibody*	Receptor/ligand	Source
ECM			
Collagen I (CI)	AB745	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$	Chemicon, Temecula
Collagen III (CIII)	3G4		Telios, San Diego, CA
Collagen IV (CIV)	CIV22	$\alpha_1\beta_1$, $\alpha_2\beta_1$	Dako, Copenhagen
Laminin B chain (LN)	4E10	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_1$	Telios
Fibronectin (FN)	3E1	$\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_4\beta_7$	Telios
Vitronectin (VN)	49.1	$\alpha_v\beta_3$, $\alpha_v\beta_5$	R&D, Abingdon, UK
Thrombospondin (TSP)	P12	$\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, CD36	Immunotech, Marseille, France
Tenascin (TN)	TN-2	$\alpha_v\beta_3$, $\alpha_9\beta_1$	Telios
Integrin			
β_1 (CD29)	4B4		Coulter, Hialeah, USA
α_1 (CD49a)	TS2/7	Laminin, collagen	FSM†, Madrid, Spain
α_2 (CD49b)	P1E6	Laminin, collagen	Telios
α_3 (CD49c)	P1B5	Laminin, collagen, fibronectin, epiligrin	Telios
α_4 (CD49d)	HP2/1	VCAM, fibronectin, thrombospondin	Immunotech
α_5 (CD49e)	P1D6	Fibronectin, thrombospondin	Telios
α_6 (CD49f)	GoH3	Laminin	Immunotech
β_3 (CD61)	SZ21		Immunotech
α_{IIb} (CD41)	SZ22	Fibrinogen, fibronectin, vitronectin, vWF‡	Immunotech
α_V (CD51)	VNR147	Vitronectin, fibronectin, thrombospondin	Telios
β_4 (CD104)	3E1		Telios

* All antibodies are mouse monoclonal, except GoH3 and AB745 which are rat monoclonal and rabbit polyclonal, respectively.
† FSM, Francisco Sanchez-Madrid.
‡ vWF, von Willebrand factor.

Tris-buffered saline plus bovine serum albumin, followed by 20 min incubation with normal horse serum. During immunostaining, sections were incubated for 1 hr at room temperature with primary antibody. Appropriate antibody concentrations were titrated in human and monkey material. Between incubations, sections were washed in Tris–saline buffer. Bound primary antibody was detected with a secondary, biotinylated antibody which was visualized by an avidin biotyn complex (ABC) peroxidase method using 3,3 diaminobenzidin (DAB) as chromogen, as described previously.[8] Slides were counterstained with haematoxylin and mounted with resin. Controls were incubated with normal mouse or rabbit IgG instead of the primary antibody. The panel of primary antibodies used is described in Table 1.

RESULTS

ECM in reactive lymph nodes and tonsils

Interfollicular and follicular areas. ECM proteins were abundantly present in the interfollicular areas, but poorly expressed in primary follicles and virtually absent in secondary follicles (Table 2). The interfollicular areas showed a strong immunostaining for collagen I and III, fibronectin and tenascin, and weaker staining for laminin and collagen IV. Vitronectin was not found in interfollicular areas, but was demonstrable in a fine reticular pattern in the apical zone of germinal centres. Furthermore, accumulation of some ECM proteins, i.e. collagen I, III and IV, fibronectin, and, to some extent, laminin, vitronectin and tenascin, but not thromospondin, was charcteristically seen at the area between mantle zones and interfollicular areas (Fig. 1).

Sinuses and fibrous capsule. The fibrous outer lymph node capsule immunostained strongly for laminin and collagen I and III, less intensively for fibronectin, vitronectin and collagen IV, but not for tenascin. In lymph nodes and tonsils with reactive hyperplasia, none of the ECM proteins studied was associated with the sinuses and sinus lining cells. In tonsils, the epithelial basal membrane immunostained abundantly for laminin, collagen IV and tenascin, and more weakly for fibronectin, vitronectin, thrombospondin and collagen III. The epithelial cells were negative for all ECM proteins except for a weak reticular pattern of collagen I between the cells.

Vessels. Vessels showed reactivity for most of the ECM proteins studied, particularly laminin and collagen I, III and IV (Fig. 2). Intrafollicular capillaries also strongly expressed fibronectin, but not thrombospondin or tenascin. The high endothelial venules (HEV) showed a similar pattern of immunostaining as interfollicular and other vessels. The ECM proteins were expressed diffusely in the vessel walls and it was difficult to differentiate between intra- and extracellular staining. All antibodies to human ECM antigens studied, except anti-tenascin, also immunostained monkey lymph nodes, with virtually the same reactivity pattern and intensity as that of human lymphoid tissues.

ECM in non-reactive lymph nodes

ECM proteins in non-reactive monkey lymph nodes showed a different pattern of distribution compared to that of monkey

Table 2. Distribution of ECM proteins in human and monkey lymphoid tissues

Histological site	CI	CIII	CIV	LN	FN	VN	TSP	TN
Primary follicle/germinal centre	++ / −	++ / −	− / −	− / −	+ / −	+ / ++	− / −	nr / −
Mantle zone	−	−	−	−	−	−	−	−
Interfollicular area (NRT)/(RT)*	++ /++	++ /++	+ /+	+ / +	+++/++	− / −	+ /+	nr /+++
Interfollicular/mantle border	+++	+++	+++	+	++	+	−	−
Fibrous capsule (NRT)/(RT)	++ /++	+++/+++	++ / +	+ /+++	+ / +	+ / +	− / −	nr / −
Sinusoids (NRT)/(RT)	+++/ −	− / −	+++/ −	− / −	++ / −	− / −	− / −	nr / −
Epithelial basal membrane†	++	++	+++	+++	+	++	+	+++
Interfollicular capillaries (NRT)/(RT)	+++/+++	+++/+++	+++/+++	+++/+++	++ / +	− / ±	++ /++	nr /+
Intrafollicular capillaries	+++	+++	+++	+++	+++	+	−	−
Paracortical HEV (NRT)/(RT)	+++/+++	+++/+++	+++/+++	+++/+++	++ /++	− / +	++ / ++	nr /++
Stromal capillaries (NRT)/(RT)	+++/+++	+++/+++	+++/+++	+++/+++	++ /+	− / +	++ / −	nr / +
Other veins, arterioles (NRT)/(RT)	+++/+++	+++/+++	+++/+++	+++/+++	+ /+	+ / ++	± / ±	nr / −

−, negative; ±, minimally expressed; +, weakly positive; ++, positive; +++, strongly positive.

* NRT, non-reactive lymph nodes. RT, reactive lymph nodes and/or tonsils. nr, antibody anti-tenascin not reactive in monkey lymphoid tissue.

† Epithelial basal membrane in tonsils.

Other abbreviations as in Table 1.

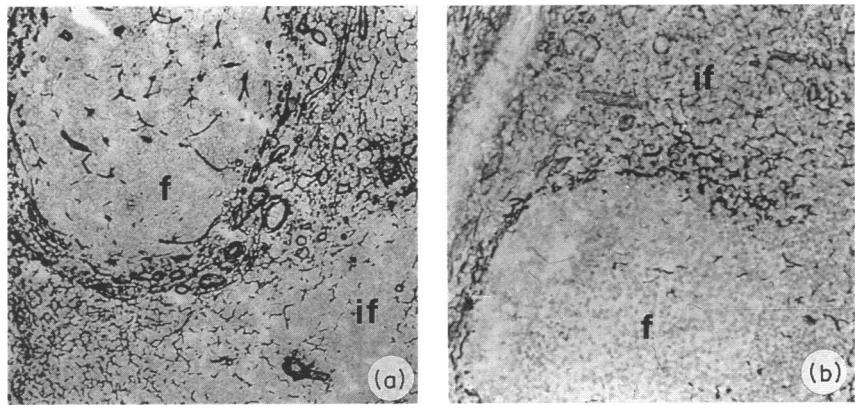

Figure 1. Immunostaining of ECM proteins in lymphoid tissues. (a) Collagen I in human tonsil (×100). (b) Tenascin in human tonsil (×100). f, follicle; if, interfollicular area. Note predominant interfollicular distribution of these ECM proteins and condensation around the secondary follicle. Vessels of the follicular and interfollicular compartments including HEV were strongly immunostained for collagen I but not tenascin.

and human reactive lymph nodes (Table 2). The major differences were as follows.

Primary follicles showed a thin trabecular immunostaining for collagen I and III, fibronectin and vitronectin, in contrast to hyperplastic germinal centres where only vitronectin was detected (Fig. 3). The reticular immunostaining displayed by vitronectin was diffuse in primary follicles, but was usually localized mostly to the apical zone of secondary follicles. No ECM proteins were demonstrable in the follicular mantle zone.

Primary follicles appeared mostly avascular, in contrast to germinal centres with numerous ECM-containing capillaries (see above).

A sinusoidal delineation (barrier) was clearly apparent in the non-reactive lymph nodes immunostained for collagen I and IV and weakly for fibronectin (Fig. 2), but not for laminin, vitronectin or collagen III. This ECM 'barrier' was not detected in the hyperplastic lymphoid tissues.

Also in the non-reactive lymph nodes, the interfollicular areas had a higher expression of ECM, particularly fibronectin compared to the follicular compartment (Table 2). Vitronectin was not demonstrable in this area.

Vessels showed in general similar immunostaining patterns and reactivity in comparison to hyperplastic tissues, with a high expression of most of the ECM proteins studied. Vitronectin was expressed weakly on some vessels and in the surrounding connective tissue as well as the outer fibrous capsule. Thrombospondin was also weakly positive in interfollicular venules including HEV.

Integrin subunits in lymph nodes and tonsils

Follicles. In reactive lymph nodes, germinal centres were weakly positive for β_1 and α_4, while the mantle zone did not express any of the tested integrin subunits (Table 3).

Interfollicular areas. β_1 and α_4 were expressed by most lymphoid cells of the interfollicular zones (Fig. 4). At the zone between follicles and interfollicular areas, β_1 was expressed in a linear pattern around the hyperplastic follicles similar to that for collagens and fibronectin (see above). No other integrin subunits were detected in this area.

Vessels. β_1, α_1, α_2, α_3, α_5 and α_6 subunits were weakly expressed on most venules and capillaries both in follicular and interfollicular compartments. A strong immunostaining in endothelial cells of venules was observed for the β_3 integrin subunit (Fig. 4). Capillaries, however, were not immunostained for the β_3 subunit, whereas α_{IIb} and α_V were usually weakly expressed in some capillary endothelial cells outside the follicles. The β_4 subunit was expressed in a few venules in

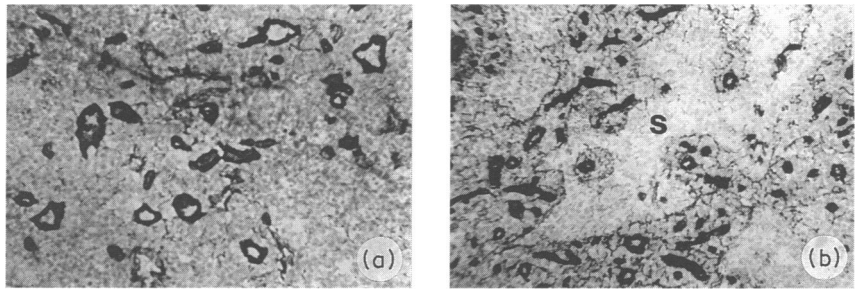

Figure 2. Immunostaining for collagen IV in vessels and sinusoids. (a) Vessels were strongly immunostained both in the follicular and interfollicular compartments of human tonsil (×200). (b) Expression of collagen IV in the sinusoidal wall of a non-reactive lymph node (×200). s, sinusoid.

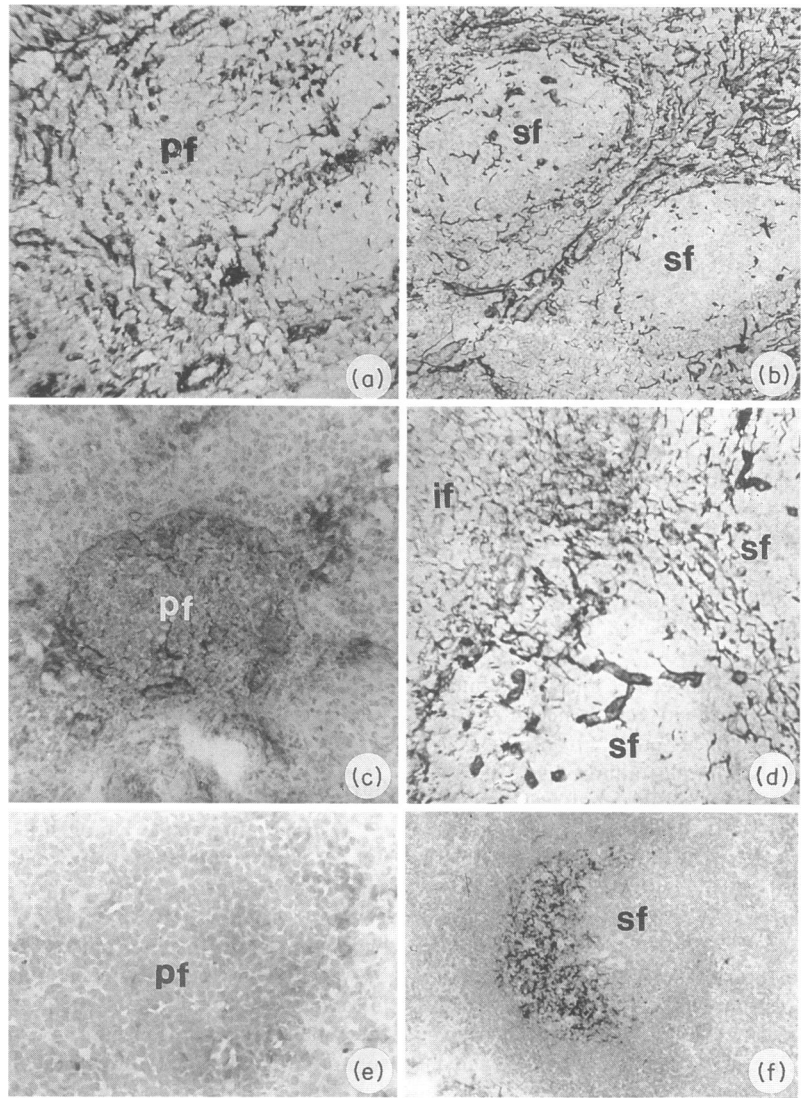

Figure 3. Comparison of expression of ECM proteins in primary and secondary follicles. (a) Collagen III in primary follicles of a non-reactive lymph node (×325), and (b) in secondary follicles in a reactive lymph node from monkey (×100). (c) Fibronectin in primary follicle of a non-reactive lymph node (×300), and (d) in secondary follicles (×160). Note the presence of both collagen III and fibronectin inside the primary follicles, and their restricted expression mainly to interfollicular areas and vessels in secondary follicles. (e) Vitronectin in the primary follicle of a non-reactive lymph node (×300) compared to (f) in the secondary follicle (×125). Note the weak expression of vitronectin in the primary follicle and the relatively strong expression in apical zones in the germinal centres. pf, primary follicle; sf, secondary follicle; if, interfollicular area.

interfollicular areas. Sinusoidal cells were not immunostained for integrin subunits. The outer fibrous capsule immunostained only for the β_1 subunit, whereas the epithelial basal membrane and epithelial cells in tonsils immunostained for β_1, β_4 and α_6 chains.

Human and monkey reactive lymphoid tissues (tonsils and lymph nodes) showed essentially the same immunostaining patterns, with minor increases in the intensity of immunostaining for α_1 and α_6 integrin subunits in monkey tissue (Table 3). In the monkey, in contrast to the human reactive lymph nodes, β_1 was clearly expressed within secondary follicles and was negative in the interfollicular mantle area of reactive lymph nodes.

Human and monkey reactive tissues showed a few differences in the expression of integrin subunits compared to non-reactive lymph nodes. Thus primary follicles were positive for β_1, whereas secondary follicles showed a weak expression of other integrin subunits (Table 3). A weak positive immunostaining of the sinusoids for β_1 and β_4 similar to that described for collagen IV and fibronectin was also present. In non-reactive lymph nodes, the outer fibrous capsule was negative for expression of integrin subunits. All endothelial cells in non-reactive lymph nodes were strongly positive for β_1, α_1 and α_6. Moreover, α_3, α_5 and particularly β_3 were well expressed on the endothelial cells of HEV, and the common venules and arterioles in the hilus.

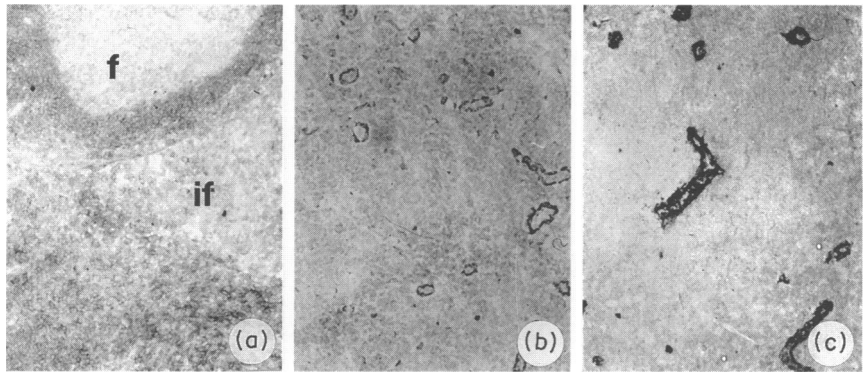

Figure 4. Immunostaining for integrin subunits in lymphoid tissue. (a) α_4 integrin subunit in a reactive lymph node (×100). f, follicle; if, interfollicular area. Note the predominant expression in interfollicular area. (b) Immunostaining for the α_6 integrin subunit showing a positive reaction in endothelial cells of all vessels (×140). (c) Immunostaining for the β_3 integrin subunit. Note the expression only in the venules (×100).

DISCUSSION

Our observations indicate distinct patterns of distribution for ECM components and corresponding integrin receptors in follicular and extrafollicular lymph node compartments. Furthermore, activation of the immune system appeared to be accompanied by a characteristic decrease in the expression of fibronectin and collagen I and III within expanding germinal centres. These observations extend previous studies and describe the virtual lack of collagen IV, laminin and corresponding receptors in germinal centres, except for small capillaries.[9–11] This suggests that cell–ECM interactions in the mantle zone and within germinal centres are not as important for the development of the germinal centre and the humoral immune response as cell–cell interactions between B lymphocytes, follicular dendritic cells (FDC) and macrophages. The ECM present in primary follicles appears to be dislocated marginally during the expansion of germinal centres, and to condense around the periphery of the follicles in a thin, membranous zone of collagen I, III and IV, laminin, fibronectin and the β_1 integrin subunit.

In contrast to the other ECM proteins studied, vitronectin was expressed both in primary and even more in secondary follicles. Its predominance in the apical zone of germinal centres may be related to the extensive trapping of immune complexes and complement on FDC in this area, as vitronectin, or S-protein, has been shown to bind to the terminal components of the complement system.[12]

The extensive and strong expression of most ECM proteins studied, particularly collagens and fibronectin in the interfollicular areas in non-reactive as well as reactive lymph nodes, is in agreement with their postulated role in controlling lymphocyte traffic in lymphoid tissues.[13] As interfollicular lymphocytes express mainly α_4 and β_1 integrin subunits, which constitute a functional fibronectin receptor, interaction of lymphocytes with fibronectin may indeed play a pivotal role in lymphocyte migration in these anatomical sites.

Tenascin, a molecule related to embryogenesis, development and tissue repair,[14] also showed a strong expression and wide distribution in the interfollicular areas. *In vitro*, tenascin has been shown to exert immunomodulatory activities, including inhibition of T-cell activation by soluble antigens, and also to alter their adhesive properties.[15] Tenascin may thus contribute to the local regulation of T-cell dependent immune responses.[16] The tenascin receptor appears to be an integrin as the cell–tenascin interaction can be inhibited with a arginine–glycine–aspartic acid (RGD) peptide,[17] and some evidence suggests that $\alpha_V\beta_3$ functions as tenascin receptor.[18]

Recirculating lymphocytes that express specific adhesion molecules can bind to HEV and thereby enter the lymph node.[19] From the present study, HEV appeared to display a similar profile of ECM proteins and integrin receptors as other vessels in the lymph node. Although fibronectin appeared less strongly expressed in HEV compared to other ECM proteins, it is suggested that it is important for lymphocyte adhesion and migration through HEV.[20] Interestingly, HEV converted to flat-walled endothelium down-regulated L-selectin ligand expression and could not mediate lymphocyte traffic, but antigen stimulation leads to a full restoration of HEV phenotype and function.[21] Obviously, expression of the tested integrins and ECM proteins is not solely responsible for the structure and function of HEV. In the present study, thrombospondin was seen in the epithelial basal membrane of tonsils, in vessels of extrafollicular areas, but not in the follicular capillaries. Its role as an adhesive molecule is controversial,[22] as it is usually expressed in inflamed and damaged tissue,[23,24] but it appears to inhibit angiogenesis and to induce proliferation arrest of cultured endothelial cells.[25,26] It is also of interest in this context that thrombospondin can be up-regulated by the tumour suppressor gene p53.[27] Thus, its presence in germinal centres would seem less compatible with a process characterized by angiogenesis and cellular proliferation. Laminin was markedly associated with all vessels in the lymphoid tissues and thereby represents a sensitive vascular marker.[29] The observed expression of ECM proteins and integrin subunits by the capillaries of the germinal centre probably reflects angiogenesis during formation of secondary follicles. However, factors governing the angiogenic response in this specialized compartment are not well characterized.[28] From the present observations all vascular endothelium, both in follicular and cortical areas, strongly expressed the β_1

Table 3. Distribution of integrin subunits in human and monkey lymphoid tissues

Histological site	β_1	α_1	α_2	α_3	α_4	α_5	α_6	β_3	α_{IIb}	α_V	β_4
Interfollicular capillaries (NRT)/(RT)	+++/+++	+++/++	+++/++	++/++	++/++	++/++	+++/+++	+++/+++	++/++	++/++	++/++
Primary follicle	+++	−	−	−	−	−	−	−	−	−	−
Mantle	− **+**	−	−	−	−	−	−	−	−	−	−
Germinal centre	± **++**	−	±	±	±	−	−	−	−	−	−
Interfollicular area (NRT)/(RT)	+++/+++	++ / +	− / +	− / +	++/++	+ /+	++ /+	++ /++	− /+	− /+	− /+
Interfollicular/mantle border	++ **−**	−	−	−	−	−	−	−	−	−	−
Sinusoids (NRT)/(RT)	+ / −	− / −	− / −	− / −	− / −	− / −	− / −	− / −	− / −	− / −	+ / −
Fibrous capsule (NRT)/(RT)	− /+++	− / −	− / −	− / −	− / −	− / −	− / −	− / −	− / −	− / −	− / −
Epithelial cells and basal membrane	+	−	−	−	−	−	++	−	−	−	++
Interfollicular capillaries (NRT)/(RT)	+++/+++	+++/++	− / +	− /++	− / −	− / +	++ /+++	− /+++	− / +	+ / +	++/++
Intrafollicular capillaries	+++	+ **++**	−	−	−	+	+ **++**	+++	+	+	−
Paracortical HEV (NRT)/(RT)	+++/+++	+++/+	+ / +	++/++	− / −	− /++	++ /+++	+++/+++	− /+	− /+	++/++
Other capillaries veins, arterioles	+++/+++	+++/++	++ / +	++/++	− / −	+ /++	++ /+++	+++/+++	− / +	− / +	+ / +
Lymphoid cells (NRT/(RT)	++ / ++	− / −	− / −	− / −	++/++	− / −	− / −	− / −	− / −	− / −	− / −
Endothelial cells (NRT)/(RT)	+++/+++	+++/++	+++/ +	+ / +	− / −	+ /++	+++/+++	+++/+++	+ / +	+ / +	++/++
Fibroblasts (NRT)/(RT)	++ /++	− /+	− /−	− / −	− / −	− / +	− / +	− / −	− / −	− / −	− / −

Abbreviations as in Tables 1 and 2.
Bold symbols indicate findings in monkeys different from humans in reactive tissue.

integrin subunit, whereas α_5 appeared to be up-regulated only in vessels of the reactive follicular and interfollicular compartments.

β_1 integrins are widely expressed in lymphoid as well as in non-lymphoid tissues.[26,29] Also, studies by flow cytometry have shown β_1 integrins on various cell types of lymphoid follicles.[30,31] By immunochemistry, α_5 expression is controversial.[32] In the present study, various α integrin subunits known to associate with β_1 appeared to be virtually absent or only minimally expressed in primary and secondary follicles, and α_4 was found on most of the interfollicular lymphoid cells. Apparently, lymphocytes express both α_4 and β_1 integrin subunits mainly in interfollicular areas, and it cannot be excluded that the β_1 integrin subunit strongly expressed in the primary follicles could be associated with α subunits other than those analysed in this study.

Our findings in lymph nodes support other studies on non-lymphoid tissues[33] showing a preferential expression of the β_3 subunit in larger vessels than capillaries, whereas α_V appeared to be weakly expressed in all vessels of interfollicular areas. This agrees with the notion that although α_V is mainly associated with β_3, it can also associate with other β subunits in different cell types such as epithelial cells.[34]

The observed characteristic, barrier-like sinusoidal co-expression of collagen I and IV, fibronectin and β_1 in the non-reactive lymphoid tissue is similar to that described for fibronectin in non-reactive lymph nodes from guinea-pigs.[35] This deposit of ECM disappeared in the reactive lymphoid tissues, possibly reflecting the increased cell traffic between the sinusoidal space and parenchyma of the lymph node. Interestingly, this sinusoidal barrier of ECM appears to undergo complete resolution, possibly mediated enzymatically by activated, migrating cells, whereas the ECM components of the primary follicles appear to be displaced to the periphery of the expanding secondary follicles.

The reactivity patterns of antibodies against ECM components and their integrin receptors in monkey lymphoid tissues were shown to correspond to those of human tissues, with the exception of tenascin. This extensive cross-reactivity indicates a high degree of ECM and integrin epitope conservation in primates, allowing studies in experimental primate models. Such studies on these and other adhesion molecules are necessary for a better understanding of the migration and specific localization of different lymphocyte subsets within lymphoid tissues, as well as the molecular histology of the immune response.

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