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Constitutive Expression of Tenascin in T-Dependent Zones of Human Lymphoid Tissues

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Tenascin is a major extracellular matrix glycoprotein that can interfere with the action of fibronectin by inhibiting cell adhesion and spreading. Although tenascin is able to exert important immunomodulatory activities on T and B cells and macrophages, little is known about its distribution in different lymphohemopoietic tissues. In this study we have analyzed tenascin immunoreactivity on cryostat and paraffin sections of normal and pathological lymphoid tissues using two different monoclonal antibodies. We demonstrated strong tenascin expression in all peripheral lymphoid tissues, whereas it was barely detectable in the thymus and in bone marrow. In reactive lymph nodes, tenascin was mainly found in T-dependent zones, forming a variably close-woven reticular network corresponding to fibroblastic reticulum cells and blood vessels basal laminae, showing a partial co-localization with fibronectin. In B-dependent zones, tenascin was restricted to blood vessels. Using double-marker analysis, we performed a thorough study comparing tenascin expression in different compartments of lymphoid microenvironments. Tenascin network appeared much thicker in chronically stimulated tissues, where CD4$^+$ lymphocytes with "memory" phenotype (CD45RO$^+$/CD45RA$^-$) were predominant, and at sites of ongoing inflammation. In particular, a striking increase of tenascin was observed in sarcoid lymph node, as well as in myasthenic hyperplastic thymuses. In addition, tenascin can be abnormally synthesized in tissue involved by various types of lymphomas, including Hodgkin's disease and hairy cell leukemia. (Am J Path 1993, 143: 1348–1355)

Lymphocytes interact with various molecular components of the extracellular matrix (ECM) through a repertoire of receptors precisely regulated according to microenvironmental stimuli to allow rapid interconversion between adhesive and nonadhesive states.[1] Many components of the integrin family are in fact receptors which are expressed after activation and can specifically recognize and bind discrete sequences on different ECM macromolecules.[2,3] In turn, some ECM molecules such as fibronectin (FN) cannot be merely regarded as cell adhesion substrata, since they have diverse important roles including mitogenic effects on immune and hemopoietic cells in cooperation with a number of cytokines and growth factors.[4,5] Since FN in the tissue is widely distributed and permanently present in excess, its adhesive and mitogenic effects are likely controlled at the level of cell receptor expression and/or by the modulation of other interfering ECM molecules. Tenascin (TN), also known as hexabrachion, cytotactin, and other names, is a disulfide-linked hexameric ECM glycoprotein characterized by a spatially and temporally restricted tissue distribution.[6–9] In different systems, TN can interfere with FN,[10,11] providing contrary functions through cell binding sites as well as antiadhesive epidermal growth factor-like repeats.[12] In addition, TN can significantly influence the shape of cells by promoting rounding and detachment, while FN increases the adhesion and spreading of cells.[9,13]

The study of TN distribution in different normal and pathological tissues can help define its actual role in

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different systems. The dramatic increase of TN observed in many epithelial tumors has suggested that it can have a significant role in the establishment of malignant activity.[14–21] In addition, TN can participate in tissue reparative processes and fibrogenesis.[22,23] Interestingly, *in vitro* TN can also exert important immunomodulatory activities on T cells, B cells, and monocytes, altering their adhesive properties. In particular, it is able to inhibit T-cell activation induced by soluble antigens.[24]

Immunohistochemical analysis has been widely used to investigate the distribution of TN in a variety of normal and neoplastic tissues,[18,19] but little data are available regarding the distribution in normal and pathological lymphohemopoietic tissues.[25] In this study, we demonstrate that TN is variably expressed in human lymph nodes, thymus, spleen, and bone marrow and describe how its distribution is altered in different neoplastic and non-neoplastic lesions affecting these tissues.

Materials and Methods

Frozen samples selected from the files of the Department of Pathology of Verona University were studied. The samples collected in the context of diagnostic and/or staging procedures included the following: 19 lymph nodes (7 aspecific lymphadenopathy, 2 toxoplasmic lymphadenopathy, 2 HIV^+ lymphadenopathy, 3 sarcoidosis, 5 Hodgkin's disease); 7 thymuses (2 normal infant thymus, 3 follicular hyperplasia in myasthenia gravis, 2 thymomas); 2 normal spleens; 2 reactive tonsils; 32 trephine biopsies of bone marrow (10 normal samples, 2 metastatic samples obtained during staging procedures for breast carcinoma, one with sarcoid granulomas, 19 with leukemia/lymphoma involvement); these latter included 4 hairy cell leukemia, 7 B-cell chronic lymphocytic leukemia, one T-cell lymphoblastic lymphoma, one common-type lymphoblastic leukemia, 2 Hodgkin's disease, and 4 follicular B-cell lymphoma.

Immunohistochemistry

Samples had been snap-frozen in liquid nitrogen and stored at −80 C. Cryostat sections were obtained and processed as described previously.[26] Immunostaining was performed following standard alkaline anti-alkaline phosphatase (APAAP) or labeled streptavidin-biotin-peroxidase (LSAB) procedures (all reagents were purchased from Dako, Carpinteria, CA).

Two different monoclonal antibodies (MAb) recognizing TN were used: 1) the commercially available anti-TN MAb (Clone TN2, IgG1, Kappa; Dako), and 2) a MAb (clone NCC-LU-45) obtained in the laboratory of Dr. S. Hirohashi, Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan. NCC-LU-45 MAb, selected from a hybridoma fusion of spleen cells of nude mice immunized by xenotransplantation with the moderately differentiated human lung adenocarcinoma cell line NCC-LU-201, binds a molecule recognized as TN by solid phase radioimmunoassay and immunoblotting analysis.

A selected panel of MAbs recognizing different lymphocyte subpopulations, accessory cells, other ECM molecules, etc. (CD1, CD3, CD4, CD8, CD11c, CD20, CD21, CD45R0, CD45RA, fibronectin, collagen IV, all purchased from Dako), were also used together with anti-TN (either on serial sections or using double-marker staining) to better evaluate the distribution of TN in different complex microenvironments. Briefly, one antibody was incubated and revealed using the LSAB system (Dako) with diaminobenzidine/H_2O_2, followed by a second antibody immunostaining revealed using the APAAP technique. Details on this double-marker technique have been described previously.[27] Serial sections from the same samples were also stained for endogenous alkaline phosphatase. The MAb TN2 was also tested on a selected number of samples conventionally fixed in formalin and embedded in paraffin using the LSAB technique.

Results

In all samples analyzed with NCC-LU-45 and TN-2 MAbs, an identical immunostaining pattern was observed on cryostat sections. Therefore, the results obtained with both reagents are described below as TN immunostaining.

Lymph node

In all samples of reactive lymph node, a consistent staining for TN was demonstrated in blood vessels as demonstrated in other immunohistochemical studies.[18,19] TN immunoreactivity appeared as a continuous staining on the walls of subcapsular and trabecular sinuses, in connection with the inner part of the lymph node capsule. T-dependent paracortical areas showed a peculiar reticular staining pattern forming small branching Y-shaped channels, connected with vascular walls (Figures 1 and 2). This pattern was similar to that revealed on sections

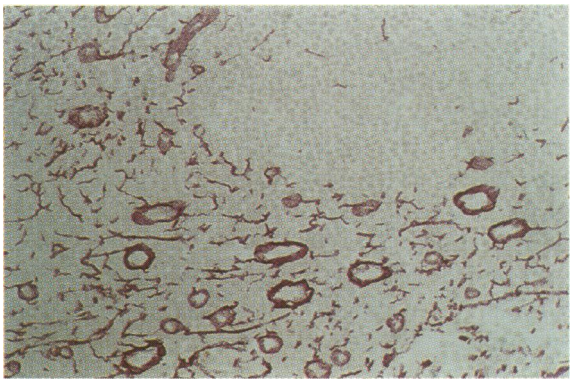

Figure 1. *Cryostat section of a reactive lymph node immunostained for TN showing a network of reticular staining connected to vascular walls. A B-cell follicle (top right) is negative. APAAP technique, ×100.*

stained for endogenous alkaline phosphatase, decorating fibroblastic reticulum cells (FRCs) and blood vessels. The density of TN^+ network varied from case to case and also in different areas of the same sample (Figure 1). A similar pattern was also observed on serial sections immunostained with anti-FN antibody. Nevertheless, FN immunostaining was much more widely expressed on capsular and trabecular structures than TN. Collagen IV immunostaining revealed a similar distribution, but with much weaker intensity on FRCs and higher intensity on vascular basement membranes and pericapsular adipocytes. In lymphoid follicles TN staining was restricted to vascular structures. In some hyperplastic follicles, a granular positivity was present within germinal centers, mainly confined to "light" zones. In paraffin-embedded lymph nodes analyzed with TN2 MAb the staining was frequently variable, with zonal heterogeneity due to differences in fixative penetration. In optimal preparations the TN immunoreactivity pattern was very similar to that observed on cryostat sections, but well defined dot-like immunostaining was also observed within the cytoplasm of endothelial cells, thus suggesting that endothelium is a major producer of lymph node TN. In double-staining preparations of TN and T-cell subset markers on cryostat sections, the localization of TN within the T-dependent microenvironment was better evaluated (Figure 2, A and B). In those lymph node samples characterized by predominance of $CD4^+$ T lymphocytes exhibiting the $CD45R0^+/CD45RA^-$ "memory" phenotype, the TN network appeared particularly prominent, but preferential association of TN^+ fibers with $CD45R0^+/CD45RA^-$ T cells was not demonstrable at the single cell level (Figure 2, A and B).

In both frozen samples of reactive tonsil the pattern of TN staining was similar to that observed in reactive lymph nodes.

In HIV^+ lymphadenopathy, characterized by predominance of $CD8^+$ T cells in paracortical areas and follicular fragmentation, TN distribution was identical to that observed in aspecific reactive lymphadenopathy, with consistent staining of FRCs and blood vessels and lack of staining in hyperplastic follicles.

In lymph nodes with toxoplasmic infection, TN distribution was similar to that observed in reactive lymph nodes.

In sarcoid lymph nodes, TN was expressed at high levels, varying from case to case and in different areas of the same sample. Concentric TN^+ thick fibrous bands were frequent around granulomas, sometimes intruding within them at sites of lymphocyte infiltration (Figure 3, A and B). The thickness and staining intensity of TN^+ bands appeared in some way related to the duration of the lesion. Thin fibrillary deposits of TN were in fact common around "young" granulomas characterized by a large number of associated $Mac387^+$ macrophages[27] and a rich $CD4^+$ T-cell perigranulomatous cuff, whereas the presence of very thick TN^+ fibrous bands was common in lymphocyte-depleted fibrotic lesions.

In lymph nodes with Hodgkin's disease, TN distribution was significantly different from that observed in hyperplastic paracortical zones. In fact, a thick, disordered meshwork of reticular staining was observed in both nodular sclerosis and mixed cellularity types (Figure 4). In some samples, this meshwork was so heavy that it covered the tissue, thus completely effacing the histological architecture.

Spleen

In agreement with a recent study,[25] in splenic white pulp TN formed a regular network of sheets around central arterioles of T-dependent zones. B-cell areas were devoid of TN, with the exception of vessel walls. In the red pulp, TN was expressed at high levels within Billroth's cords, mainly associated with reticular cells, and in sinus walls arranged to form banded structures similar to ring fibers. The overall staining pattern was different from collagen IV and CD8 immunostaining.[28]

Thymus

In normal infant thymus, TN immunoreactivity was restricted to thin linear bands corresponding to thymic capsule and blood vessels in cortical and medullary zones (Figure 5A). Perilobular adipocytes did

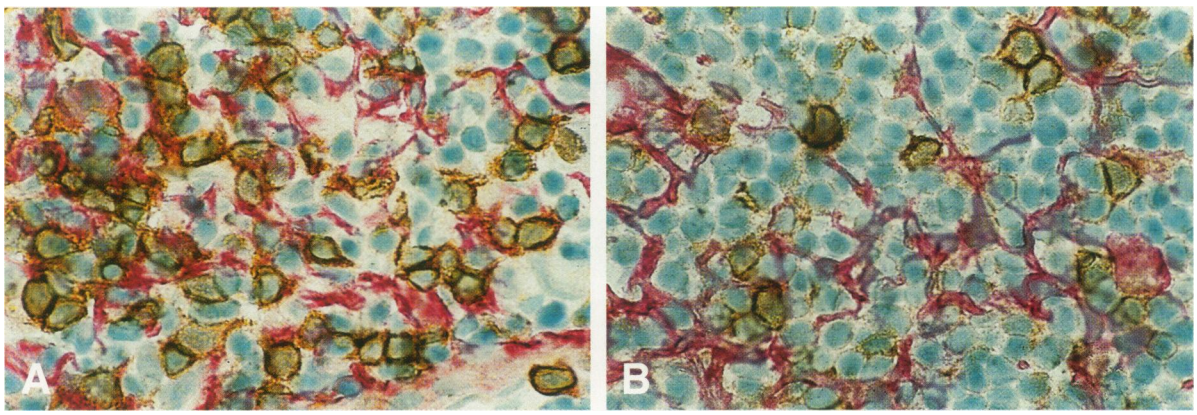

Figure 2. *Cryostat sections of a reactive lymph node immunohistochemically analyzed using double-marker technique (see text) to evaluate the relationships between TN expression and T-cell subset (CD45R0, CD8) distribution. Anti-TN MAb was revealed using the APAAP technique (red), while CD45R0 (A) and CD8 (B) were revealed using the LSAB technique (brown), ×1000.*

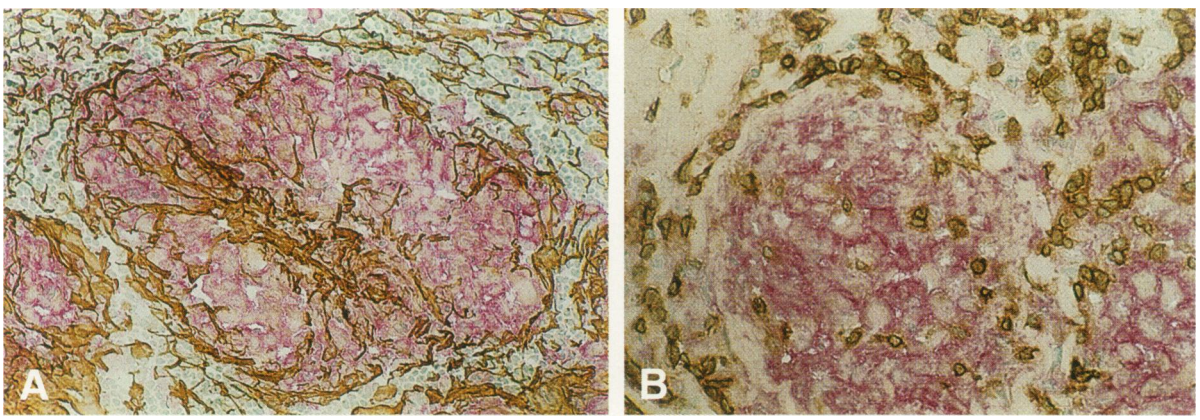

Figure 3. *A: Cryostat section of a sarcoid lymph node showing thick TN$^+$ fibrous bands (brown) around and within a CD11c$^+$ sarcoid granuloma, ×100. B: A consecutive section of the same granuloma immunostained for CD3 (brown) and CD11c (red). LSAB/APAAP double marker technique, ×250.*

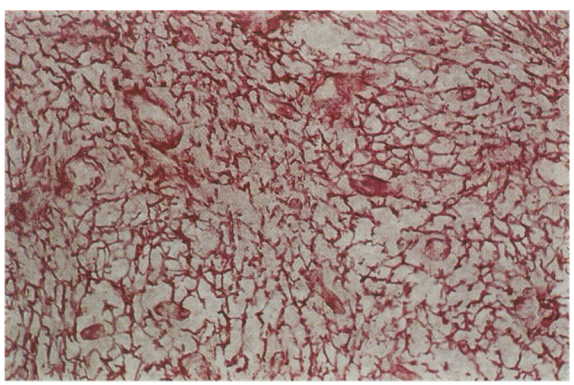

Figure 4. *Cryostat section of a lymph node from a patient with Hodgkin's disease characterized by the presence of a thick, disordered meshwork of reticular TN staining. APAAP technique, ×250.*

not express significant amount of TN. Serial sections of the same specimens immunostained for collagen IV showed a different pattern, with strong expression on adipocyte basement membranes.

In samples of hyperplastic thymus from myasthenic patients, the pattern was strikingly different. In these samples, lymphatic follicles with germinal centers were included in large T-cell rich infiltrates characterized by a TN$^+$ network identical to that observed in reactive lymph node paracortical areas (Figure 5B). This pattern of staining allowed a clear-cut distinction between unaffected cortical and medullary areas and inflamed areas.

In thymomas, as observed in normal thymus, TN expression was confined to vessels and, when present, to dilated perivascular zones (Figure 5C). Areas of medullary differentiation, when present, were mainly devoid of TN immunostaining.

Bone marrow

In normal human bone marrow trephine biopsies, TN expression was restricted to vessel walls and endosteal cells lining the bone trabeculae. A light and irregular staining was evident in interstitial spaces

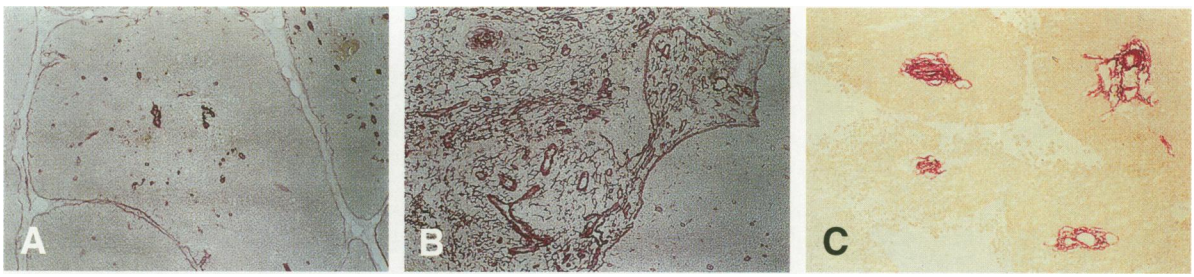

Figure 5. *A: Cryostat section of normal infant thymus showing a low level of TN expression restricted to thymic capsule and blood vessels, ×40. This pattern is strikingly different from that observed in hyperplastic thymus from a myasthenic patient (B) where a dense TN+ network characterizes the inflamed areas infiltrated by peripheral T lymphocytes. A lymphatic follicle is present exhibiting granular TN immunoreactivity (top left), ×40. C: Cryostat section from a thymoma showing strong TN immunoreactivity confined to dilated perivascular spaces. APAAP technique, ×40.*

and adipocytes (Figure 6A). This pattern was clearly different from that observed using anti-collagen IV antibody, which clearly decorated the adipocyte basal membranes. In paraffin-embedded samples, strong immunoreactivity for TN was evident within and around bone trabeculae, corresponding to osteocytes and bone lining cells. In these preparations, a dot-like intracytoplasmic immunostaining was observed in a variable number of mononuclear cells, which were characterized as erythroid precursors by glycophorin immunostaining.

In the sample obtained from a patient with sarcoidosis, strong immunoreactivity was observed around granulomas.

All bone marrow samples with localization of hematological neoplasms showed a significant increase of TN expression in affected areas. This was mainly determined by a variable increase of the number and thickness of interstitial fibers, which did not parallel in all cases the conventional reticulum defined by silver staining. In all the samples of hairy cell leukemia, extracellular accumulation of TN was particularly intense (Figure 6B), as was also the case in samples with Hodgkin's disease and in the two bone marrow metastases of breast carcinoma at the epithelial/mesenchymal interface.

Discussion

In this paper, we provide evidence that TN is an important component of the ECM in T-dependent zones of peripheral lymphoid tissues including lymph node, tonsil, and spleen. In fact, in paracortical lymph node zones, as well as in interfollicular areas in the tonsil and in periarteriolar zones of splenic white pulp, strong TN immunostaining was demonstrated forming a canalicular network linked to blood vessels identical to that formed by FRCs. On the other hand, bone marrow and thymus, central lymphohemopoietic tissues, are normally characterized by very low levels of TN expression, mainly confined to blood vessels. In both these tissues the amount of TN deposition is dramatically increased in different reactive lesions. In myasthenic patients with "thymitis" the peripheral-type lymphoid tissue intruding the thymic medulla[29] is directly responsible for the increase in TN deposition. In the

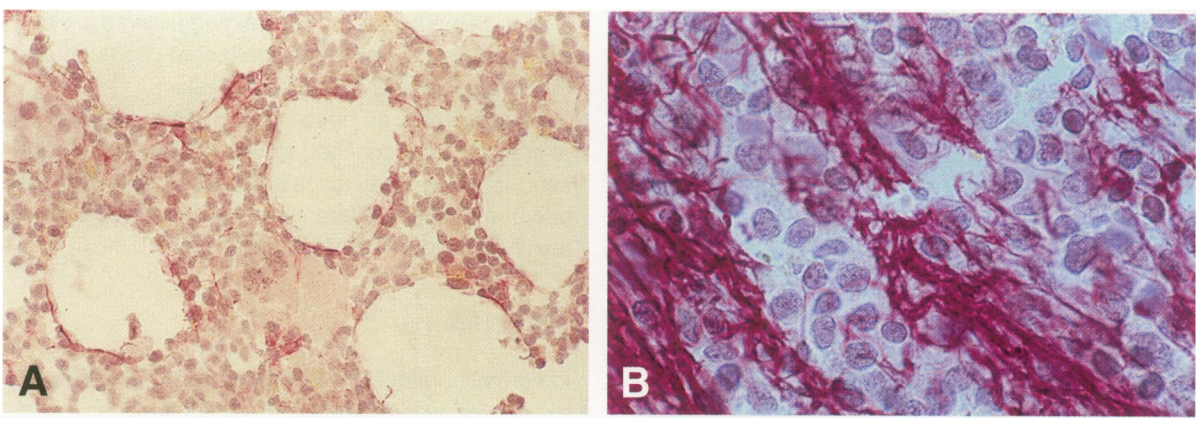

Figure 6. *A: Cryostat section of trephine biopsy from a normal bone marrow showing light and irregular TN immunoreactivity, ×250. This pattern is strikingly different from that observed in a bone marrow sample (B) from a patient with hairy cell leukemia where thick TN+ reticular fibers are admixed to neoplastic hairy cells. APAAP technique, ×1000.*

bone marrow most reactive and neoplastic lesions are characterized by a significant enhancement of the interstitial amount of TN.

According to our immunohistochemical results, TN expression is heterogeneously modulated in different lymphoid tissues, with enhanced accumulation in lymph nodes exhibiting features of "activation" as defined by the presence of expanded T-cell areas with many high endothelium venules and the prevalence of T lymphocytes exhibiting the $CD45R0^+/CD45RA^-$ memory phenotype. The increase of TN network in our study was particularly evident in lymph node samples harboring ongoing inflammation, such as in sarcoidosis.

These findings are important for a number of reasons. First, the large amount of TN associated with FRC can significantly contribute to the function of this poorly understood component of T-dependent microenvironment. FRCs constitute a supportive cellular framework in T-dependent microenvironments, and may also help in the binding and migration of antigenic material as well as in the regulation of lymphoid traffic through the modulation of a variety of contractile and adhesive molecules.[30,31] TN has been shown to exert significant immunomodulatory activities, including the inhibition of T-cell activation induced by soluble antigens and alloantigens, altering their adhesive properties.[24] The demonstration of constitutive expression of large amounts of TN in paracortical lymph node areas, at sites of T-cell activation and proliferation, suggests that this peculiar ECM molecule can significantly contribute to the local regulation of immune responses after antigen challenge. T lymphocytes upon activation can change the repertoire of adhesion molecules on their membranes, acquiring the "mobile" phenotype necessary for leaving the lymphoid tissues to seek specialized tissues such as gut mucosa and lung.[32] This phenomenon is particularly evident in sarcoid lymph nodes, where, in spite of enhanced proliferation,[33] the total number of T cells (and especially the unprimed T-cell population) is progressively depleted, with increase of tissue areas effaced by granulomas.[34] The accumulation of large amounts of TN associated with basement membranes and fibroblastic stroma can provide activated memory T cells with the repulsive microenvironment necessary for their migration out of the lymph node.[35] As observed in other tissues, TN and FN were co-localized in T-dependent areas. On this basis, it is likely that the proportion and position of these two molecules can be relevant in modulating the actual adhesive or repulsive attitude of immune cells with ECM and FRCs, as well as the proliferative or inhibitory effect on naive and memory T cells.[5,24]

In vitro studies have recently demonstrated that TN synthesis and secretion are significantly increased by transforming growth factor-β (TGF-β),[36] a multifunctional factor with potent regulatory effect on the development and organization of ECM.[37] TGF-β is produced and secreted by a variety of cell types including activated T lymphocytes and macrophages.[38,39] In Hodgkin's disease, where abnormal production of TGF-β has been demonstrated,[40,41] we observed a striking increase in the disordered organization of the TN reticular framework. It is possible to speculate that in Hodgkin's disease the excessive deposition of TN, together with other microenvironmental abnormalities,[30,42] can contribute to the deregulation of cell-mediated immunity commonly observed in this disease.

In normal and pathological bone marrow samples, the variation of TN immunoreactivity was particularly evident. The low level of TN expression observed in all normal samples was strikingly different from most neoplastic cases, where variable enhancement of immunostaining was found. Previous studies were mainly focused on the induction of TN synthesis provided by epithelial-mesenchymal interactions. To our knowledge, this is the first report showing that TN can be induced in the bone marrow involved by hematological neoplasms. Further studies are needed to verify whether the abnormal accumulation of TN can interfere with the normal development of blood precursors in the marrow microenvironment, and whether it is due to factors produced by neoplastic cells, as demonstrated in epithelial neoplasms[43,44] or by intervening reactive cells.

Another finding of interest is the difference of TN expression observed between normal thymus and lymph node paracortical zones and thymic samples with hyperplastic modifications in autoimmune disease. Although thymic medulla and lymph node paracortex share some relevant similarities, including the prevalence of lymphocytes with mature phenotype ($CD3^+$, $CD4^+$ or $CD8^+$, $CD1^-$, TdT^-) and the presence of antigen presenting interdigitating cells,[45] thymic medulla is unique in providing the peculiar microenvironment where negative selection of autoimmune T-cell clones takes place.[46,47] Alterations of TN expression, as observed in myasthenic thymuses in our study, could have a role in the mutation of adhesive properties of thymocytes to other relevant microenvironmental medullary components thus contributing to tolerance breakdown.[48]

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