Renal subcapsular tertiary lymphoid aggregates in chronic kidney diseases

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Abstract
In the last decade, it has been accepted the formation of tertiary lymphoid organs in the renal parenchyma during inflammatory conditions. These organized cellular aggregates contain B- and T-lymphocytes, dendritic cells, surrounded by neo-lymphatic vessels. They have been described in renal allografts, acute and chronic interstitial nephritis, IgA and membranous nephropathies. The functional characteristics of these lymphoid nodules remained still under consideration. After investigating the renal biopsies of 268 patients with primary and secondary nephropathies, we have selected 20 cases showing lymphoid-like cellular aggregates located just beneath the renal capsule and having close contacts with this kidney envelope. All of these cases also showed an associated medium sized lymphatic vessel. The ultrastructure of these nodules proved to contain more or less the same cellular composition: lymphocytes, dendritic cells, seldom plasma cells and macrophages. We consider these particular subcapsular lymphoid-like nodules to be tertiary lymphatic structures in close association with the perirenal lymphatics, and the first to develop in any type of inflammatory and autoimmune renal condition.

Keywords: kidney, tertiary lymphoid organs, chronic nephropathies, diabetes, neoangiogenesis.

Introduction
Recent data have indicated that lymphoid neogenesis may play a role in maintaining immune responses against persistent antigens, and thus promoting chronic inflammation [1]. The aim of an immune response is to destroy a certain pathogen. Nevertheless, there are such resistant pathogens due to some efficient countermeasures to the host immune reaction or because the antigen is self or tumoral, capable of inducing tolerance, and resulting in a lasting immune response, featured as a chronic inflammation. It is well known that tissues hosting a chronic inflammation process are stuffed with effectors cells like T-cells, macrophages, dendritic cells, B and plasma cells organized as secondary lymphoid organs with follicles and T-cell areas. This event has been termed lymphoid neogenesis or tertiary lymphoid organ (TLO) formation [2, 3].

Therefore, the lymphoid neogenesis has focused growing attention as well as the list of conditions involving TLO comprises autoimmune diseases, chronic inflammatory diseases, infections and some tumors. Histopathological and immunohistochemical analyses indicate that lymphoid neogenesis is a dynamic process during which lymphocytic infiltrates develop into aggregates that eventually organize in secondary follicles with germinal centers and T-cell areas [3, 4]. The most organized structures were found in highly infiltrated organs, which indicate that TLO induction requires extensive local activation of immune cells. Although different patterns of lymphoid arrangements usually coexist in the same patient, individual cases with rheumatoid arthritis tend to have the same type of synovial lymphoid infiltrate, indicating a role for host factors in TLO induction [5]. The basic cellular constituents of B- and T-cell areas of a TLO are similar to a SLO (secondary lymphoid organs). Nevertheless, the overall organization of the first differs markedly from the conventional one of the second. Unlike lymph nodes, it seems that TLOs are not supplied by afferent lymph vessels and not encapsulated [6]. It remains also to be determined weather TLOs also lack the intricate canalicular system of conduits and corridors, which in TLOs regulate the lymph flow and the traffic of antigen-presenting cells, lymphocytes and chemokines to specific areas of lymphoid tissues [7].

Most of the conditions in which lymphoid neogenesis has been documented are organ-specific autoimmune disorders in which both B- and T-cell responses against tissue antigens have been implicated. Thus, lymphoid neogenesis is most prominent in thyroid autoimmune diseases [8] and myasthenia gravis [9]. Lymphoid neogenesis probably evolved as part of a strategy to contain chronic local infections [10].

Gastritis induced by Helicobacter pylori shows a high degree of TLO organization [11]. The lymphoid neogenesis is also a common feature in chronic hepatitis C-virus infection (HCV) [12].

Small kidney biopsy series described a percentage of 2–27% with interstitial leukocytes as being B-cells [13]. Using CD20 as a B-cell marker it has been described prominent accumulations of these cells in the interstitial involvement in IgA and membranous nephropathies [13, 14]. These B-cells were observed either as diffuse infiltrates, or as TLO. B-cells were located mostly in the center of TLOs reminding the follicles in lymph nodes. These aggregates were also surrounded by neolymphatics [15]. Although the cellular elements are the same as in lymph nodes, TLOs differ in that they do not have afferent lymphatics, nor a fibrillar stroma or capsule [16].
The initiation of an inflammatory response in kidney may occur by activation of tubular epithelial cells or resident dendritic cells (DC). Thus, some DCs travel to the regional lymph nodes activating and priming lymphocytes for the specific response. These primed lymphocytes are next recruited into the tubulo-interstitium. Some of these DCs may not receive cues to traffic out of kidney after taking up an antigen. Thus, renal DCs may release the antigen in the interstitial compartment of kidney. In chronic antigen stimulation conditions, lymphocytes encounter these DCs and may initiate the formation of lymphoid-like follicles [16]. The inflammatory response may promote the detection of tubulointerstitial antigens as autoantigens, leading to a persisting interstitial inflammatory response. Therefore, a chronic interstitial inflammation might pave the way for a misguided immune response in which interstitial antigens might be detected as "dangerous".

The present work started some time ago after noticing that some kidney biopsies showing interstitial inflammation, displayed TLOs in the subcapsular periphery of the organ, in contact with the fibrous capsule.

Materials and Methods

A series of 268 renal biopsies have been investigated for diagnosis. The diagnostics covered almost the whole non-tumoral kidney pathology, and involved both primary and secondary nephropathies.

Routinely all kidney samples were harvested with guillotine biopsy needles of 16G. One or two renal punctures have been performed in each case in order to get enough tissue for immunofluorescence (IF), transmission electron microscopy (TEM) and paraffin embedding when possible. As a rule, the two ends of each fragment (1 mm) were placed in sodium cacodylate buffered glutaraldehyde (pH 7.3) for EM. Thus, if the biopsy sample started from the kidney surface, the most peripheral 1 mm block was glutaraldehyde fixed and embedded in Epon for EM. The middle part of the fragment was covered with cryo-gel embedding medium and frozen in a Leica cryostat for IF. For the routine diagnostic we used nine monospecific antibodies, FITC conjugated (anti-IgA, IgG, IgM, Fibrinogen, Albumin, C3c and C1q complement fractions, Kappa and Lambda light chains).

The glutaraldehyde fixed fragments (4–24 hours) were washed overnight in cacodylate buffer, dehydrated in a graded ethanol series and finally embedded in Epon.

When tissue available for light microscopy was obtained, the paraffin embedded sections were stained with HE, PAS, and silver methenamine.

Results

The diagnostic statistics of our series of 268 kidney biopsied patients is emphasized in Table 1.

The figure corresponding to each pathologic entity showed just its frequency among the patients with clinical indication for biopsy, and not its general impact in population.

Table 1 – Diagnostic statistics of the 268 patients after kidney biopsy examined with LM, IF and EM

<table>
<thead>
<tr>
<th>No.</th>
<th>Nephropathies</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>IgA nephropathy</td>
<td>39</td>
</tr>
<tr>
<td>2.</td>
<td>Minimal change disease</td>
<td>32</td>
</tr>
<tr>
<td>3.</td>
<td>Membranous nephropathy</td>
<td>26</td>
</tr>
<tr>
<td>4.</td>
<td>Focal segmental glomerulonephritis</td>
<td>24</td>
</tr>
<tr>
<td>5.</td>
<td>Lupic nephropathy</td>
<td>23</td>
</tr>
<tr>
<td>6.</td>
<td>Renal amyloidosis</td>
<td>18</td>
</tr>
<tr>
<td>7.</td>
<td>Diabetic nephropathy</td>
<td>16</td>
</tr>
<tr>
<td>8.</td>
<td>Membrano-proliferative glomerulonephritis</td>
<td>10</td>
</tr>
<tr>
<td>9.</td>
<td>Tubulo-interstitial nephropathy</td>
<td>9</td>
</tr>
<tr>
<td>10.</td>
<td>Post-infectious acute nephropathy</td>
<td>8</td>
</tr>
<tr>
<td>11.</td>
<td>Thin GBM disease + Alport syndrome</td>
<td>7</td>
</tr>
<tr>
<td>12.</td>
<td>Kidney transplant associated lesions</td>
<td>4</td>
</tr>
<tr>
<td>13.</td>
<td>Vasculitis</td>
<td>3</td>
</tr>
<tr>
<td>14.</td>
<td>Myeloma cast nephropathy</td>
<td>3</td>
</tr>
<tr>
<td>15.</td>
<td>Anti-GBM disease</td>
<td>1</td>
</tr>
<tr>
<td>16.</td>
<td>Hemolitic-uremic syndrome</td>
<td>1</td>
</tr>
<tr>
<td>17.</td>
<td>Others, including normal aspects and lack of glomeruli</td>
<td>44</td>
</tr>
<tr>
<td>TOTAL patients 268</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

More then half (51.8%) of biopsy samples showed different intensities and distribution of interstitial inflammation. As a rule, the inflammation was diffuse, and sometime associated with nodular lymphocytic aggregates. About one third of these biopsy samples showed in one small block a fragment of the kidney capsule and the adjacent renal tissue. In twenty cases, these subcapsular renal blocks included a cellular aggregate of mostly lymphocytes, plasma cells and dendritic cells. The shape of these aggregates was generally rounded having a diameter between several hundreds and one thousand microns. These sizes were mostly dependent on the level of sectioning, in the center or in periphery of the nodule.

The 20 patients found with subcapsular lymphocytic aggregates were diagnosed as shown in Table 2.

Table 2 – The frequency of nephropathies in the 20 biotopic samples found with subcapsular lymphocytic aggregates

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosed nephropathies</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>IgA nephropathy</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>Membranous nephropathy</td>
<td>4 (stage II – 2; stage III–IV – 2)</td>
</tr>
<tr>
<td>3.</td>
<td>Focal segmental glomerulosclerosis</td>
<td>3</td>
</tr>
<tr>
<td>4.</td>
<td>Minimal change disease</td>
<td>2</td>
</tr>
<tr>
<td>5.</td>
<td>Membrano-proliferative glomerulonephritis type I</td>
<td>2</td>
</tr>
<tr>
<td>6.</td>
<td>Diabetic nephropathy</td>
<td>2 (class III – 1; class IV – 1)</td>
</tr>
<tr>
<td>7.</td>
<td>Tubulo-interstitial nephropathy</td>
<td>2</td>
</tr>
<tr>
<td>8.</td>
<td>Lupic nephropathy</td>
<td>1 (class III)</td>
</tr>
<tr>
<td>TOTAL patients with subcapsular TLO 20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The highest frequency was in patients with membranous and IgA nephropathies, and next in focal and segmental glomerulosclerosis.

These lymphocyte aggregates were placed either next to the renal capsule, or in its immediate proximity, and were not accompanied by deeper similar structures (Figure 1, A and B). The cells composing these nodules
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were tightly packed to each other and not surrounded by any kind of capsule. Sometimes the aggregates enclosed renal tubules (Figure 1C) and superficial glomeruli also. The aggregates were constantly accompanied by wide opened vessels. Upon the walls of these vessels, the lack of red blood cells, and their white blood cells content, we considered to belong to the lymphatic vascular system. The walls and their caliber were similar to the lymphatic collecting vessels of nodes. These large vessels were placed either immediately beneath the fibrous capsule (Figure 1A), or deeper in the aggregates (Figure 1, B and D).

The cell population of aggregates was set up mainly of lymphocytes, a few plasma cells, macrophages and dendritic cells. We did not find any germinal center in all our cases. The aggregates had no fibrous capsule; they were surrounded by the cortical structures of renal parenchyma. The main part of the cells was typical lymphocytes: oval nuclei, little cytoplasm with a high density of free ribosomes and few mitochondria (Figure 2). From place to place, we found dendritic cells (DC) having rounded or shaggy nuclei, lighter than those of lymphocytes, and a branched, less dense cytoplasm. The cytoplasmic processes contain ribosomes, mitochondria and dilated rough endoplasmic cisterns (Figure 3). Beside the cytoplasmic processes of DCs we found clusters of collagen fibers and extracellular matrix. Macrophages were also present (Figure 4), but no polymorphonuclears have been frequently seen.

Some problems occurred in differentiating the many vessels crossing the aggregates. We clearly found lymphatic capillaries filled with lymphocytes (Figure 5). The walls of these capillaries were set up of very thin endothelial cells endorsed by a discontinuous basement membrane and a sparse layer of collagen fibers. The presence of lymphocytes in the lumen rendered visible these capillaries, hardly to be identified otherwise. Definite blood capillaries were also present (Figure 6). The endothelial layer containing Weibel–Palade bodies was well defined. Basement membrane was continuous. The lumina displayed red blood cells and were more or less opened. Some blood capillaries with closed lumina gave the impression of neocapillaries (Figures 7 and 8). As a whole these lymphocytic organized structures are supported by a fibrous stromal framework set up of collagen type III fibers, vessels and extracellular matrix. The large opened collecting vessels displayed walls

![Image](image-url)
with poorly demarcated layers, similar to venules and housed many features of cell migration. Some lymphocytes were placed beneath the vessel basement membrane, between the basement membrane and the pericytes (Figure 9) and others between the basement membrane and the thin endothelial cytoplasmic veil (Figure 10). In the first case, lymphocytes were surrounded by a halo of a transparent extracellular space (Figure 9), while in the second case there was a close contact between lymphocytes and endothelial cells (Figure 10).

Different to other lymphoid organs we could not identify high endothelial venules.

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**Figure 2** – Subcapsular lymphocytic aggregate. Lymphocytes (Ly), dendritic cell (DC), macrophage (Mf), thin septa of collagen fibers and extracellular matrix (S).

**Figure 3** – Stellate shaped dendritic cell (DC) surrounded by lymphocytes (Ly). In between these cells, it can be noticed type III collagen fibers (Col). The cytoplasm of DC contains free and attached ribosomes, dilated rough endoplasmic reticulum (RER), mitochondria.

**Figure 4** – Lymphocytic aggregate showing densely packed cells (left side), clusters of collagen fibers organized as septa (col.), fibroblasts (Fb), a macrophage (M) and a lymphatic vessel (V).

**Figure 5** – Lymphatic capillary vessel bordered by thin endothelial cells (En) and clusters of collagen fibers (col). The lumen contains four lymphocytes (ly).

**Figure 6** – Blood capillary vessel. The wall is structured by endothelial cells (En) showing Weibel–Palade bodies and a well-defined basement membrane (bm).

**Figure 7** – Blood capillary vessel with an almost closed lumen (L). Endothelial cell (En) with elaborate cytoplasm containing mitochondria, ribosomes and Weibel–Palade bodies surrounded by a thin basement membrane (bm) and interstitial collagen fibers.
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Discussion

The concept of tertiary lymphoid organs (TLO) occurrence during chronic nephropathies with immunopathologic background has been well established during the last decade [17–22]. Nevertheless, two facts are differentiating the above-mentioned TLOs from those described in the literature, and thus making a particular type of subcapsular lymphocytic aggregates. First, is their location in the very close proximity of the renal capsule, and the second is the ubiquitous presence of large lymphatic collector vessels. The kidney capsule is set up of smooth muscle cells, type III collagen fibers and seldom fibroblasts. This capsule is not an impenetrable structure. Thus, although we did not encounter lymphatic vessels crossing the capsule, the close contact between these subcapsular TLOs and the renal capsule (Figure 1) suggests a possible connection between the extra-capsular and the renal parenchyma lymphatic networks.

The small size of the 1 µm thick sections makes difficult the observation of subcapsular lymphocytic aggregates (400–1000 µm). If we take into consideration the size of the needle biopsy sample, which do not exceed 1300 µm in diameter, one can imagine how weak are chances to find them unless they are very frequent. Beside all these difficulties, another must for being visualized is that the biopsy sample should intercept the renal capsule attached to the periphery of kidney parenchyma. Thus, the search of these subcapsular TLOs becomes a rather hard task unless they are developed in large number.

Our statistics shows the occurrence of subcapsular TLOs during the chronic evolution of primary and secondary nephropathies having more or less known immunopathologic backgrounds, with the IgA and membranous nephropathies heading twice more frequent than the others. Since these two diseases are generally the most frequent in any statistics, we consider this aspect as no relevant. We believe that this event depends more readily on the chronic evolutionary stage of each nephropathy.

The general picture of cells setting up these aggregates was quite simple resembling that of lymph nodes follicles. The majority were typical lymphocytes having uniform features, most probable B-cells since they were intermingled with dendritic cells. The immunohistochemical typing of these lymphocytes should be a most difficult task since human renal biopsy samples of this particular subcapsular area are not available for research. The dendritic cells showed the same aspect as the follicular dendritic cells (FDC) of lymph nodes mantle zone. Renal dendritic cells (rDCs) are major constituents of the mononuclear phagocytic system in normal kidneys [24]. The rDCs derive from a common bone marrow macrophage and have blood
monocytes as circulating precursors [25]. Experimental evidence is mounting that rDCs help to maintain peripheral tolerance in the kidney at steady state [26]. Any non-self antigens introduced into normal kidneys should be captured by rDCs and presented in draining renal lymph nodes, leading to immune activation and proliferation of antigen-specific CD4+ T-lymphocytes [27]. New studies have demonstrated renal compartment-specific accumulation of rDC subsets during renal inflammation. BDCA1+ conventional and BDCA4+ plasmacytoid rDCs accumulate in the renal interstitium [28]. DC-SIGN+ conventional rDCs occur in abundance, along with BDCA2+ plasmacytoid rDCs in the renal interstitium [29]. TLOs composed of CD21+ follicular rDCs and CD20+ B-cells have been detected, suggesting a conserved immunologic program executed by DCs, whether resident or recruited [16].

B-cells in human kidney are part of the inflammatory cells in various diseases: acute and chronic interstitial nephritis, renal allografts, IgA and membranous nephropathies. B-lymphocytes are almost exclusively located in the tubulointerstitium and not in the glomeruli. Intrarenal B-cells can be part of a local system to enhance the immunological response by functioning as antigen presenting cells, and as a source for cytokines promoting T-cell proliferation and lymphatic neoangiogenesis [16]. It has been suggested that treatment with Rituximab, a chimeric antibody to the CD20 molecules expressed on B-cells may be a potential therapeutic mechanism.

Macrophages were seldom, and related to apoptotic lymphocytes. Fibroblasts and the connected collagen fibers constitute a honeycomb type framework, which sustains the lymphocytic aggregate.

The occurrence of these subcapsular TLOs also involves the neoformation and differentiation of blood and lymphatic vessels [23], a process connected with the B-cells secreted cytokines. Regarding the identification in TEM of the lymphatic vessels, this can be done either if their lumina are open, or they are filled with lymphocytes. The wall layers of TLO lymphatics are either if their lumina are open, or they are filled with lymphocytes. While the larger collecting vessels have some pericytes, the ubiquitous presence of a large open lymphatic collector capsule, in apposition with this kidney envelope. The intense lymphocytes migration through the walls of local vessels proves its involvement in the renal autoimmune conditions.

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References

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