T-Cell Mediated Inflammatory Activity in the Stellate Ganglia of Patients with Ion Channel Disease and Severe Ventricular Arrhythmias

Running title: Rizzo et al.; T-cell ganglionitis in ion-channel diseases

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Journal Subject Codes: [5] Arrhythmias, clinical electrophysiology, drugs
Abstract:

**Background** - Long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) are electrical diseases characterized by catecholamine-induced ventricular arrhythmias. Unbalanced autonomic innervation of the heart may trigger arrhythmic events and stelllectomy is a treatment option for patients who are resistant to pharmacological drugs. We analysed left stelllectomy specimens of LQTS and CPVT patients for signs of inflammatory activity.

**Methods and Results** - Stellate ganglia were retrieved from 12 consecutive patients (8F, 4M, mean age 23.4±17 yrs) with either LQTS (n=8) or CVPT (n=4) and serious arrhythmias. Control stellate ganglia were obtained from 10 accidently deceased patients (6F, 4M, mean age 35±17.6 yrs). Sections were immunostained with antibodies against T cells (CD3, CD4, CD8, CD20, Granzyme B), CD68 (macrophages) and HLA-DR antigens (activation marker). Immunopositive cells were quantified as cells/mm². PCR and RT-PCR were performed to screen for herpes virus DNA. Stellate ganglia of all 12 LQTS/CVPT patients revealed mild but distinct inflammatory infiltrates composed of T-lymphocytes and macrophages, which were diffusely spread, but also clustered in small foci apposed to ganglion cells, interpreted as T cell-mediated ganglionitis. Morphometrical analysis showed that CD3+ and CD8+ T cells/mm² were significantly higher in the ganglia of LQTS/CVPT cases than in healthy controls (p=0.0018 and p=0.0009, respectively). Molecular analyses were negative for neurotropic viruses.

**Conclusions** - T-cell mediated cytotoxicity towards ganglion cells may boost adrenergic activity as to trigger or enhance electrical instability in LQTS/CVPT patients who are already genetically predisposed to arrhythmias.

**Key words:** sympathetic nervous system, pathology, inflammation, catecholaminergic polymorphic ventricular tachycardia, long QT syndrome
Introduction

Long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) are primary electrical diseases characterized by catecholamine-associated ventricular arrhythmias manifested as syncope and sudden cardiac death (SCD) in the absence of any structural cardiac pathological substrate. The pathogenesis of these diseases is complex. Mutations in genes encoding ion channels (LQTS)\(^1\,^2\) or genes involved in the calcium homeostasis (CPVT)\(^3\,^6\) can be detected by genetic screening of patients. Both LQTS, typically 1 and 2 subtypes, and CPVT characteristically present with emotional- and exercise-induced syncope and/or SCD. A link to autonomic imbalance has been established and ventricular arrhythmias have been associated with the level of sympathetic activation. Studies by Schwartz et al\(^7\) identified increased sympathetic tone as trigger for arrhythmic events in many patients with LQTS, and unbalanced innervation of the heart with a left-sided sympathetic predominance. Pharmacological \(\beta\)-adrenergic receptor antagonists are used to counteract the actions of the sympathetic neurotransmitter; however, they have several side effects and are not always sufficiently effective. Left cardiac sympathetic denervation (LCSD), as an additional therapy to the use of \(\beta\)-blockers for the treatment of malignant arrhythmias in LQTS, was proposed for the first time in 1971 by Moss et al\(^8\), as done previously in ischemic heart disease\(^9\,^10\). At present permanent removal of the sympathetic inputs to the heart is considered a safe and effective strategy for high-risk patients as primary and secondary prevention in ion channel diseases\(^11\,^14\). LCSD seems to decrease the excitability through prolongation of ventricular refractoriness, thus reducing propensity to ventricular fibrillation (VF) due to an increase of VF threshold\(^15\,^16\). The mechanisms by which autonomic tone influences the arrhythmic risk and the pathology of cardiac innervation, both intrinsic and external to the heart, are not known. Since pathological investigations on the sympathetic
innervation of the hearts of these patients are scarce, we evaluated the stellate ganglia of LQTS/CPVT patients with resistant arrhythmias for the occurrence of structural abnormalities. Specifically we investigated the occurrence of inflammatory activity and the type of immune response with the use of immunohistochemical techniques. In addition, PCR and RT-PCR were used to study the presence of genomic sequences of neurotropic viruses within the stellate ganglia of these patients.

Methods

Study Population

Surgically excised left stellate ganglia and thoracic ganglia T2 to T4 were retrieved from 12 consecutive patients (8F, 4M, age range 2-54, mean age 23.4±17 yrs) with either LQTS (n=8) or CVPT (n=4) who underwent LCSD at one referral center (Academic Medical Center, Amsterdam, the Netherlands). The diagnosis was based on conventionally reported criteria. Details of the patients are summarized in Table 1. All LQTS and CPVT patients were under maximum beta-blockers therapy and had persistent serious arrhythmia events. Genotype of the patients was available in all cases, except 1. Control stellate ganglia were obtained from 10 accidently deceased patients (6F, 4M, age range 20-64, mean age 35±17.6 yrs). The study was approved by the local ethics committee, and informed consent was obtained from each patient.

LCSD procedure

The LCSD was performed as previously described in detail. Briefly, LCSD involves ablation of the lower half of the left stellate ganglion, together with the thoracic ganglia T2 to T4 through a videoscopic transthoracic approach. It provides adequate cardiac denervation leaving the upper stellate ganglion intact to minimize the risk of Horner syndrome.
Histology/immunohistochemistry

Tissue samples were fixed in 10% buffered formalin and paraffin-embedded; 5 μm-thick sections were stained with hematoxylin-eosin (HE) and Masson’s trichrome to evaluate the presence and extension of fibrosis.

For immunohistochemical studies, the following antibodies were used: polyclonal anti-S100 (general neuronal marker, DAKO, Glostrup, Denmark) to visualize the neural network, monoclonal anti-CD3 (Pan-T, clone SP7, Thermo Scientific/LabVision, Fremont, CA, USA), monoclonal anti-CD4 (T-helper lymphocytes, clone 4b12, Thermo Scientific/LabVision, Fremont, CA, USA), monoclonal anti-CD8 (Cytotoxic T-cells, CTL’s, clone SP16, Thermo Scientific/LabVision, Fremont, CA, USA); monoclonal anti-Granzyme B (activated CTL’s, clone GRB-7, Monosan, Sanbio, Uden, the Netherlands), monoclonal anti-CD20 (mature B-cells, clone L26, DAKO, Glostrup, Denmark) and monoclonal anti-CD68 (macrophages, PG-M1, Dako, Glostrup, Denmark), to analyze the distribution and the lineage of inflammatory populations in ganglionic tissues. In addition, monoclonal HLA-DR antibodies (clone CR3/43; DAKO, Glostrup, Denmark) were used as markers for up-regulation of antigen presentation and T-cell activation. All antibodies were applied in a Streptavidin biotin complex (SABC) method with DAB as a substrate to visualize immunoreactivity.

Negative controls consisted of sections incubated following the same procedure, but in the absence of the primary antibody.

The absolute number of cells that stained with CD3, CD20, CD4, CD8 antibodies was measured with the use of computer-assisted morphometric analysis (Image Pro Plus image analysis software (Media Cybernatics, Bethesda MD, USA), in all cases and controls. The numbers of cells were expressed as median number of cells/mm2 (minimum-maximum).
Slides were examined independently by two histopathologists (ACW and SR), blinded to group allocation and clinical characteristics. All morphology studies (light microscopy and immunohistochemistry) were performed on stellate ganglia and thoracic ganglia T2 to T4.

Molecular studies

PCR and RT-PCR analysis for detection of herpetic viral genomes was performed on frozen tissue. Total RNA and DNA were extracted from homogenates of entire sections using phenol chloroform methods. Briefly, reduced frozen specimens were re-suspended in two different digestion solutions: one for RNA and one for DNA extraction. The nucleic acids were extracted using a modified Chomczynsky and Sacchi’s method. Qualitative and quantitative evaluation of the nucleic acids was performed using spectrophotometer. The oligonucleotides used to ascertain the quality of extracted RNA or DNA were complementary to the mRNA glyceraldehyde-3-phosphate dehydrogenase (3GPDH) and β-globin gene, respectively. PCR was used to evaluate DNA viruses (cytomegalovirus-CMV, herpes simplex virus-HSV, Epstein-Barr virus-EBV, human herpes virus 6-HHV6, varicella zoster virus-VZV). The RT-PCR for the late gene of CMV was used to detect not only the presence of CMV but also its replicative form. The PCR products were analyzed by electrophoresis on a 3% Nu-Sieve agarose containing ethidium bromide and photographed under ultraviolet transillumination.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) or median (minimum-maximum). CD3, CD8, CD4 and CD20 were compared between groups with Wilcoxon rank sum test. Statistical significance was set at the 5% level. We calculated the 95% confidence interval (95% CI) for the prevalence of ganglionitis in cases with the exact method.
Results

Histological findings

Histological examination with standard HE staining revealed inflammatory cells infiltration and degenerative changes of ganglion cells with vacuoles within the cytoplasm in all LQTS/CPVT cases (prevalence=1.00, 95% CI 0.74-1.00).

Mononuclear inflammatory cells were diffusely spread, but also clustered in small foci in close apposition to ganglion cells (Fig. 1). There were no neutrophils or eosinophils present. In two controls, only scattered lymphocytes were observed, in the absence of clusters in close apposition to ganglion cells. There were no inflammatory changes in the peri-ganglionic fat tissues of LQTS/CPVT cases and controls. Inflammatory changes were accompanied by mild fibrosis in both LQTS/CPVT cases.

Immunohistochemistry

Immunohistochemical analysis demonstrated a diffuse infiltration with CD68+ macrophages and CD3+ T cells in all LQTS/CPVT cases. The largest fraction of CD3+ T-cells were CD8+ CTL’s, of which many contained GranzymeB+ granules (a marker of activation) and HLA-DR expression, and clustered around some of the ganglion cells (Figg. 2, 3). These clusters also contained macrophages, showing abundant expression of HLA-DR antigens. At these sites, aberrant expression of HLA-DR was also noticed in the adjacent affected ganglion cells.

Quantitative analysis of inflammatory cells in stellectomy specimens and surrounding tissues revealed that the density (cells/mm²) of CD3+ and CD8+ T-cells in the LQTS/CPVT ganglionic samples was significantly higher than in the adjacent fat tissue, and also significantly higher than in control ganglia of the 10 accidentally deceased persons.
Specifically, the median number/mm² of CD3+ T-cells was 40 (9-180) in cases vs. 13.95 (7-20) in control ganglia (p=0.0003), and of CD8+ CTL’s was 32.5 (8.7-147.0) in cases vs. 12.2 (6-16) in controls (p=0.0001), i.e. 81% and 87% of CD3+ T-cells, respectively. In contrast, the number of CD4+ T-helper cells and CD20+ B-cells was very low: 0.25(0.00-8.10) and 0.73 (0.00-3.57), respectively, similar to that of controls (Table2).

No difference was found when comparing ganglion-related T-cells of LQTS and CPVT patients: CD3+ 34.8 (9-180) vs. 47 (38-60) (p=0.3677), CD8+ 27 (8.7-147) vs. 36.3 (32-48) (p=0.4828).

Molecular analysis

Ganglion tissue was screened for the presence of herpes viral DNA in order to determine the potential trigger for the immune cell infiltration. The ganglia were assessed for the occurrence of CMV, HSV-1 and HSV-2, EBV, HHV6, VZV. No viral DNA was present in homogenates of ganglion entire sections of LQTS/CPVT cases and controls.

Discussion

Left cardiac sympathetic denervation raises the ventricular fibrillation threshold 24 and this represents the rationale for the use of stellectomy to reduce or prevent malignant arrhythmias typical of LQTS and CPVT13.

In our series of 12 consecutive patients with either LQTS or CPVT who underwent LCSD, the resected stellate ganglia revealed signs of a chronic ganglionitis, characterized by an elevated number of activated T- lymphocytes and degeneration of adjacent ganglion cells. These inflammatory changes were found in all patients, and independently of gender, age, onset of the disease and genetic substrate. All patients had persistent serious arrhythmia events despite
maximum beta-blockers therapy. Normal ganglia also displayed some inflammatory activity, but to a significantly lower extent.

Intracardiac ganglionitis and its potential effect on arrhythmic risk have been previously described in LQTS subjects who died suddenly\textsuperscript{25-27}. Ganglionitis and neuritis in the sinus node area were found also in liquid-protein-modified-fast diet dieters who developed prolongation of the QT interval and ventricular arrhythmias after dieting\textsuperscript{28}. In these studies, diffuse infiltration of mononuclear cells associated with neural degeneration was found at histological examination, lacking however of an immune-characterization of the cellular composition and activity of the inflammatory population. By means of immunohistochemistry, we demonstrated a predominant CD3+ CD8+ CTL’s infiltrate in close proximity of macrophages and high expression of class II MHC antigens in the ganglionic specimens. Such an inflammatory pattern can be considered as the immunohistological “footprint” of the involvement of an (auto) immune-mediated disorder or viral diseases, and makes a nonspecific inflammatory reaction in the ganglia unlikely. Moreover, several of the lymphocytes surrounding ganglion cells were stained for Granzyme B, a serine protease expressed in the cytotoxic granules of lymphocytes implicated in triggering apoptosis in target cells\textsuperscript{29}. The recruitment of CD8+ CTL’s may be due to the aberrant expression of HLA-DR by ganglion cells, but also immune reaction itself can induce MHC class II antigen expression in the nervous system\textsuperscript{30}.

The most frequently proposed theories suggest either a viral\textsuperscript{31} and/or autoimmune etiology, but also paraneoplastic, toxic or neurological disorders may underlie the onset of an inflammatory process in the ganglia via neurotransmitters, growth factors, and cytokines.

The similarity of the histological abnormalities in all our patients suggested an infectious disease as the most probable hypothesis, since ganglionitis appears mainly in the presence of
neurotropic viral infections such as Herpesviridae. These viruses share the ability to establish a latent infection in the ganglia and can be reactivated during life\textsuperscript{32}. CD8+ CTL’s are known to localize at the sites of herpetic lesions to prevent reactivation from latency\textsuperscript{33}.

In our LCSD specimens, many ganglion cells were indeed surrounded by CD8+ CTL’s, but none of the stellate ganglia contained HSV-1, HSV-2, or VZV DNA. Therefore, it remains unresolved why T-cells surround apparently not infected ganglion cells in our series. It cannot be excluded with certainty that these neurons host other viruses or minimal amounts of viral antigen which are not detectable with the techniques applied in the present study. Finally, many ion channels also express in neurological tissue; as a speculation, it could be that aberrant ion channel function dysregulates the homeostasis in the ganglia leading to a secondary “sterile” inflammation.

However, it is fair to state that at present, the origin of the inflammatory infiltrates in ganglia, and more specifically, whether or not they might be causally related to the primary electrical diseases of the heart remains unsettled. Despite this, we suppose that the presence of active ganglionic inflammation, albeit low grade, can be of importance for the symptomatology of such patients. From our observations it can be speculated that ganglionic inflammation might contribute to the cardiac electrical instability in subjects with LQTS/CPVT, and particularly in those who are heavily symptomatic.

In conclusion, small foci of active chronic inflammation with affinity for the ganglion cells in the excised stellate ganglia of all 12 patients with LQTS/CPVT who underwent LCSD support the hypothesis that T-cell mediated cytotoxicity towards ganglion cells may boost adrenergic activity, and thus may trigger or enhance electrical instability in these patients who are already genetically predisposed to arrhythmias. Ganglia removed from not affected
individuals also displayed a mild inflammation. This could imply that ganglionic inflammation is functionally important only in symptomatic patients who are genetically predisposed to arrhythmias and remains unnoticed in most other individuals.

**Limitations**

The study population is a small number (12 patients), but relatively large in a genotyped juvenile population. Within the limitations of small numbers, the data show differential features between LQTS/CPVT patients and controls, suggesting a “triggering role” of inflammation for the sympathetic nervous system.

**Funding Sources:** Stefania Rizzo was a visiting fellow at the Academic Medical Center, Amsterdam, the Netherlands, supported by the Registry of Cardio-Cerebro-Vascular Pathology, Veneto Region, Venice, Italy.

**Conflict of Interest Disclosures:** None.

**References:**


ryanodine receptor (RyR2) gene in familial polymorphic ventricular tachycardia. *Circulation.* 2001;103:485-490


Table 1 Clinical data, genetic and histomorphometric data of the study population.

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<tr>
<th>Pt N.</th>
<th>Sex</th>
<th>Age</th>
<th>Clinical picture</th>
<th>ICD</th>
<th>Mutation</th>
<th>Histo-Morphometry</th>
<th>Outcome</th>
<th>Follow-up (months)</th>
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<td>1</td>
<td>F</td>
<td>20</td>
<td>CPVT</td>
<td>-</td>
<td>RyR2:</td>
<td>CD3 38/mm²</td>
<td>Alive, free of symptoms</td>
<td>83</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>Glu4076Lys</td>
<td>CD8 32/mm²</td>
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<td>2</td>
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<td>2</td>
<td>LQTS8</td>
<td>+</td>
<td>CACNA1C:</td>
<td>CD3 180/mm²</td>
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<td>3</td>
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<td>4</td>
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<td>Alive, rare recurrence of appropriate ICD shock</td>
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<td>R1623Q</td>
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<td>4</td>
<td>F</td>
<td>17</td>
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<td>-</td>
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<td>90</td>
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<td>F</td>
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<td>+</td>
<td>KCNH2:</td>
<td>CD3 9/mm²</td>
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<td>Alive, free of symptoms</td>
<td>83</td>
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<td></td>
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<td>G785V</td>
<td>CD8 32/mm²</td>
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<td>7</td>
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<td>47</td>
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<td>-</td>
<td>KCNQ:</td>
<td>CD3 19/mm²</td>
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<td>48</td>
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<td></td>
<td></td>
<td>Arg595Gln</td>
<td>CD8 16.5/mm²</td>
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<td>37</td>
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<td>Thr613Met</td>
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<td>6</td>
<td>CPVT (not RyR)</td>
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<td>CD3 42/mm²</td>
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<td>45</td>
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<td>KCNQ1:</td>
<td>CD3 49/mm²</td>
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<td>36</td>
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<td></td>
<td>Ala341Val</td>
<td>CD8 40/mm²</td>
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<tr>
<td>11</td>
<td>F</td>
<td>21</td>
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<td>-</td>
<td>RyR2:</td>
<td>CD3 52/mm²</td>
<td>Alive, significantly less ventricular arrhythmias</td>
<td>13</td>
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<tr>
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<td>M</td>
<td>54</td>
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<td>+</td>
<td>KCNH2:</td>
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<td>9</td>
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<td>Arg582Cys</td>
<td>CD8 79/mm²</td>
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* Many stands for >10; † Patient 2 died of severe brain damage due to intractable arrhythmias that occurred during surgery

Abbreviations: ICD=Implantable Cardioverter Defibrillator; NSVT=Non-Sustained Ventricular Tachycardia; PVB Premature Ventricular Beats; VT=Ventricular Tachycardia
**Table 2:** Morphometrical analysis of immuno-response in the stellectomy specimens of arrhythmia patients (LQTS/CPVT) and controls.

<table>
<thead>
<tr>
<th>T-cell subsets/mm²</th>
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<th>Controls (n=10)</th>
<th>p-value</th>
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<td>CD3</td>
<td>40 (9-180)</td>
<td>13.95 (7-20)</td>
<td>0.0003</td>
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<td>CD8</td>
<td>32.5 (8.7-147.0)</td>
<td>12.2 (6-16)</td>
<td>0.0001</td>
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<td>CD4</td>
<td>0.25 (0.00-8.10)</td>
<td>1.27 (0.11-3.59)</td>
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<td>CD20</td>
<td>0.73 (0.00-3.57)</td>
<td>1.27 (0.12-2.35)</td>
<td>0.2604</td>
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</table>

**Figure Legends:**

**Figure 1** Stellate ganglion in a patient with CPVT (A, D), LQTS (B,E) and a control (C,F).

A, B: inflammatory cells, predominantly lymphocytes, are seen in clusters around the cytoplasm of the ganglion cells; in B, note also mild fibrosis (hematoxylin-eosin); C Scattered mononuclear cells in a control stellate ganglion (hematoxylin-eosin); D, E,F: CD3 immunostaining of A, B,C, respectively; (scale bar A, B, C 20 μm; D, E, F 40 μm)

**Figure 2** Immunophenotypical characterization of the inflammatory infiltrate in a CPVT patient (pt#1). A: Panoramic view (CD3, scale bar 50 μm); B-F: close-up of a cluster around the ganglion cells (B: CD3; C: CD20; D: CD8; E: HLA-DR; F: Granzyme B). The majority of lymphocytes were CD3+ CD8+ T cells.

**Figure 3** Immunophenotypical characterization of the inflammatory infiltrate in a LQTS patient (pt#12). A: Panoramic view (CD3, scale bar 50 μm); B-F: close-up of a cluster around the ganglion cells (B: CD3; C: CD20; D: CD8; E: HLA-DR; F: Granzyme B). Lymphocytes mostly consisted of CD3+ CD8+ T cells.
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