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sCD163 in AOSD: a biomarker for macrophage activation related to hyperferritinemia

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Abstract Ferritin has a key role in Adult-onset Still's disease (AOSD). Its production seems related to macrophage activation of which sCD163 is a major serum marker. Thus, we aimed at evaluating the role of sCD163 in AOSD and its relationship with ferritin. Furthermore, we determined the expression of CD163 and ferritin in a lymph-node from an AOSD patient. sCD163 and serum ferritin were measured in 34 patients with AOSD (21 active, 13 non-active), 18 sepsis and 22 healthy controls (HC). Immunohistology was performed on a lymph-node from an AOSD patient in order to detect CD163 and ferritin. A tonsil from an HC was used as control. Mean sCD163 (8.6 ± 5.4 mg/L) was higher in active AOSD than "non-active" patients (4.6 ± 2.7 mg/L, $p = 0.02$). The mean sCD163 in AOSD (6.9 ± 4.9 mg/L) and sepsis (7.1 ± 5.6 mg/L) were higher than in HC (2.56 ± 1.17 mg/L, $p < 0.001$), but no difference between AOSD and sepsis was detected. sCD163 positively correlated with ferritin ($p = 0.0045$; $r = 0.4755$) only in AOSD. Serum ferritin (mean 3,640.1 ± 6,896.9 μg/L) was higher in active AOSD than in sepsis (1,720.2 ± 3,882.1 μg/L, $p < 0.007$). CD163 was equally distributed in the B and T areas of both lymph-node and tonsil. Differently from the tonsil, ferritin was expressed only in the lymph-node B area. sCD163 is a marker of disease activity in AOSD. The correlation with ferritin may lead to hypothesize a macrophage activation related to hyperferritinemia. Ferritin was found expressed only in the B area of the AOSD lymph-node, suggesting a role for this molecule as an antigen in the disease pathogenesis.

Keywords Ferritin · sCD163 · Macrophage · Adult-onset still's disease

Introduction

Ferritin is a key protein in iron metabolism, mainly being involved in iron detoxification and iron storage. Recently, a new possible role for this molecule in the pathogenesis of different autoimmune and auto inflammatory conditions has been hypothesized [1]. Indeed, four immune-mediated conditions characterized by increased ferritin serum levels were gathered under the single nomenclature "Hyperferritinemic Syndrome": macrophage activation syndrome (MAS), Adult-onset Still's disease (AOSD) [2], catastrophic antiphospholipid syndrome (cAPS) [3] and septic shock [4]. In AOSD, hyperferritinemia is currently used as a key diagnostic tool. AOSD is a rare inflammatory condition with a protean clinical picture characterized by spiking fever, maculopapular rash, arthritis and variable systemic features [5]. Concerning AOSD laboratory findings, it is characterized by a marked neutrophilic leukocytosis, increased serological markers of inflammation and increased cytokine production including IL-18 which is one of the main cytokines driving the inflammatory response [6, 7]. In this condition, ferritin serum values are up to five times or more above the upper limits of normal, reaching in some cases really massive levels (>50,000 μg/

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L). In order to diagnose AOSD, a fivefold increase in serum ferritin has been suggested to have a specificity and sensitivity of 41 and 80 %, respectively [8]. In a recent paper, Lian et al. [9] combined the most commonly used diagnostic criteria for AOSD, the Yamaguchi criteria, with hyperferritinemia (identified by a cutoff of 2,500 μg/L) obtaining a specificity for AOSD diagnosis of 99.9 % [10]. Thus, serum ferritin level has long been proposed as a predictor for AOSD diagnosis as well as a biomarker to monitor the disease activity and treatment response.

The precise mechanism of ferritin production in AOSD has still to be clarified. A possible role for the histiocyto-macrophagic system and/or an increased release from damaged hepatocytes has been proposed. Recently, Cohen et al. [11] demonstrated ferritin production from macrophages through a non-classical secretory pathway in a mouse model. Macrophages represent pivotal cells in the regulation of iron homeostasis. During inflammatory states, they are characterized by an increased iron uptake and a repressed iron release [12]. The sCD163 is a molecule nearly exclusively expressed on cells of monocytic lineage, and it is released by shedding into the sera during macrophage activation [13]. Thus, sCD163 is considered a marker of macrophage activation.

The aim of our study was to evaluate sCD163 expression in active and non-active patients with AOSD and its possible use as biomarker for the disease. Moreover, we aimed at identifying an eventual correlation between sCD163 and ferritin serum levels in order to support the macrophagic origin of this molecule. Finally, we evaluated the tissue expression of these molecules on a lymph-node of a patient with active AOSD in order to characterize the expression and the localization of both ferritin (L and H chains) and CD163 in the inflammatory tissue.

Methods

Patients

Consecutive patients diagnosed with AOSD (according to the Yamaguchi criteria) [9] in our rheumatologic unit were enrolled. Disease activity was evaluated with the Rau's criteria [14]. In this set of criteria, the total score ranges from 0 to 12 and is calculated through the addition of one point assigned to each symptom: fever, evanescent rash, pleuritis, pneumonia, pericarditis, hepatomegaly or abnormal liver function tests, ferritin serum levels $\geq$3,000 μg/L, lymphadenopathy, WBC >15,000/mm^3, sore throat, myalgias and arthritis. Patients with a Rau's score $\geq$4 were considered active. As control groups, healthy controls (HC) and consecutive patients with sepsis (ACCP/SCCM Consensus Conference Criteria) [15], who referred to our primary care unit, were recruited. All patients and controls gave written informed consent. This study was approved by the local "Sapienza" University of Rome ethical committee.

Serological analysis

In all patients and subjects, ferritin serum levels were detected by chemiluminescence, LIAISON Immunoassay (DiaSorin). The levels of the sCD163 were determined by ELISA (IQ Products; the Netherland).

Immunohistochemical analysis

Immunohistochemical analysis was performed on a paraffine-embedded lymph-node from a patient with AOSD in order to detect CD163 (anti-CD163 mAb-1 Mouse, Fisher Scientific), ferritin light chain (Anti-Ferritin Light Chain polyclonal Ab Rabbit, ABCAM) and ferritin heavy chain (Anti-Ferritin Heavy chain polyclonal Ab Rabbit, ABCAM).

- Staining for anti-ferritin heavy and light chains antibodies was performed on sequential sections of a lymph-node biopsy from an AOSD patient in order to evaluate the expression and localization of these molecules. The same analysis was performed on a tonsil tissue from a healthy patient as a positive control. Paraffin-embedded sections measuring 3 μm in thickness were dewaxed and rehydrated. All sections underwent high temperature antigen retrieval using citrate buffer solution (pH 6.0) for 45 min at 95 °C (Dako, Cambridge, UK). Peroxidase and Protein blocking (Dako, Cambridge, UK) were carried out on all sections in order to prevent background binding for 10 min each, and PBS washings were performed in between blockings. Sections were stained using the primary antibodies anti-ferritin heavy and light chains for 1 h, respectively. Sections were rinsed in PBS and incubated for 30 min at room temperature with an anti-rabbit EnVision system. After washing, color reaction was developed using DAB solution (Dako), slides counterstained with hematoxylin, dehydrated and mounted in DePex.
- Further, lymph-node sections underwent the same process described above using the primary antibody anti-CD163 for 1 h. After rinsing in PBS, incubation for 30 min at room temperature with an anti-mouse EnVision system was performed. After washing, color reaction was developed using DAB solution (Dako), slides counterstained with hematoxylin, dehydrated and mounted in DePex.

Statistical analysis

Differences in quantitative variables were analyzed by the Mann–Whitney *U* test when comparing two groups, and by

the Kruskal–Wallis with Dunn's posttest when comparing multiple groups. χ^2 test with Yates' correction when required or Fisher's exact test when appropriate were used to evaluate associations of qualitative variables in the different groups. All the statistical analyses were performed using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software). A two-tailed p value of less than or equal to 0.05 was considered statistically significant.

Results

Thirty-four patients with AOSD (Female = 22, 64.7 %, Male = 12, 35.2 %, mean age ± SD = 36.08 ± 16.2 - years, mean age at onset ± SD = 32.2 ± 14.6 years), 18 patients with sepsis (*Female* = 8, 44.4 %, *Male* = 10, 55.6 %, mean age ± SD = 54 ± 17.4 years) and 22 healthy subjects were enrolled. Twenty-one/34 (61.7 %) AOSD patients were active at the moment of blood drawing according to Rau's criteria. Mean ferritin serum levels and sCD163 levels in patients with AOSD (active and non-active) and sepsis are reported in Table 1. Mean sCD163 levels ± SD in HC was 2.56 ± 1.17 mg/L. Mean serum level of sCD163 in AOSD and sepsis was significantly higher when compared to HC ($p < 0.001$; Fig. 1). In AOSD, a significant difference in sCD163 levels was detected between active and non-active patients ($p = 0.02$; Fig. 2). No significant difference was detected between active AOSD and sepsis ($p = 0.3$; Fig. 3). A significant difference in mean ferritin serum levels was found between active (5,691.5 ± 8,177.3 μg/L) and non-active patients with AOSD (325.6 ± 308 μg/L; $p = 0.039$). Mean ferritin serum level in patients with active AOSD was significantly higher compared with patients with sepsis ($p < 0.007$). sCD163 serum levels positively correlated with ferritin serum levels ($p = 0.0045$; $r = 0.4755$) only in AOSD patients (Fig. 4).

By immunohistochemical analysis, CD163 was equally expressed in the B and T cells areas of both the lymph-node of the AOSD active patients and the tonsil of the HC (Fig. 5a, b). Both ferritin H and L subunits were highly expressed only in the B cell area of the AOSD lymph-node (Fig. 5d, f), while it was possible to find both subunits widely expressed not only in the B cell area but also in the T area of the control tonsil (Fig. 5c, e).

Table 1 Mean sCD163 and ferritin serum level in patients with AOSD and Sepsis

Patients (number)	sCD163 mg/L (mean ± SD)	Ferritin μg/L (mean ± SD)
AOSD ($n = 34$)	6.9 ± 4.9	3,640.1 ± 6,896.9
Active AOSD ($n = 21$)	8.6 ± 5.4	5,691.5 ± 8,177.3
Non-active AOSD ($n = 13$)	4.6 ± 2.7	325.6 ± 308.0
SEPSIS ($n = 18$)	7.1 ± 5.6	1,720.2 ± 3,882.1

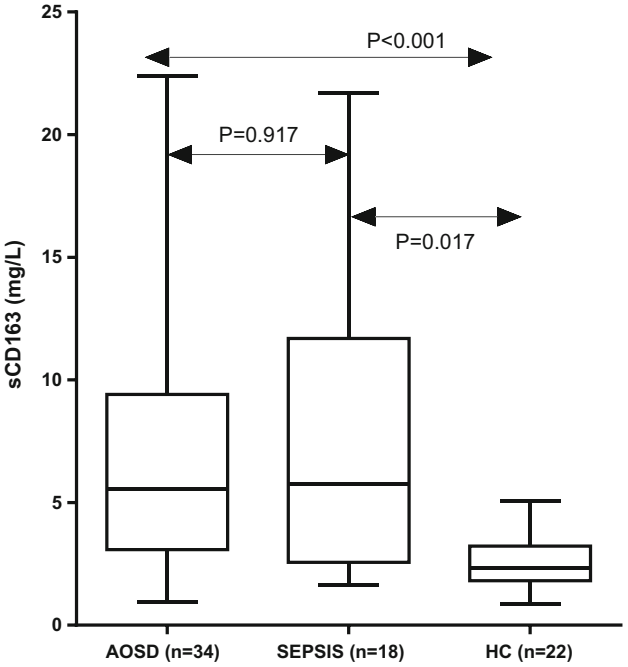

Fig. 1 Comparison between sCD163 serum levels in patients with AOSD, sepsis and HC

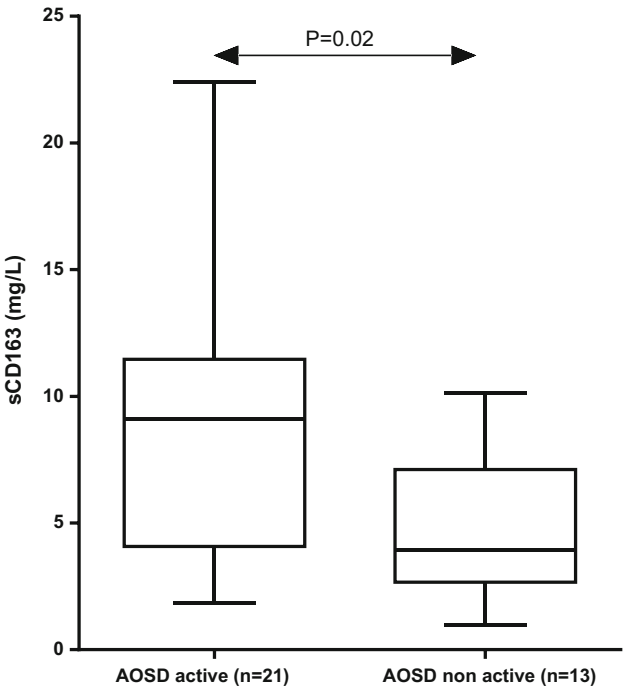

Fig. 2 Comparison between sCD163 serum levels in active versus non-active patients with AOSD

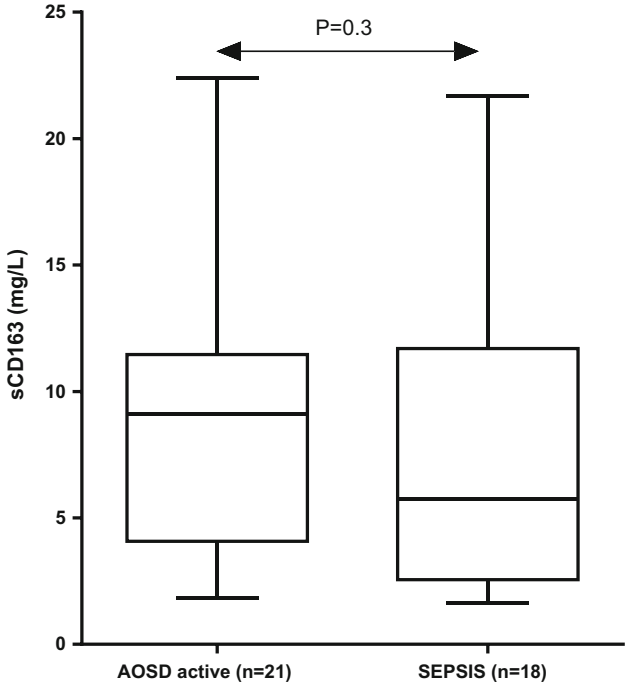

Fig. 3 Comparison between sCD163 serum levels in active AOSD versus patients with sepsis

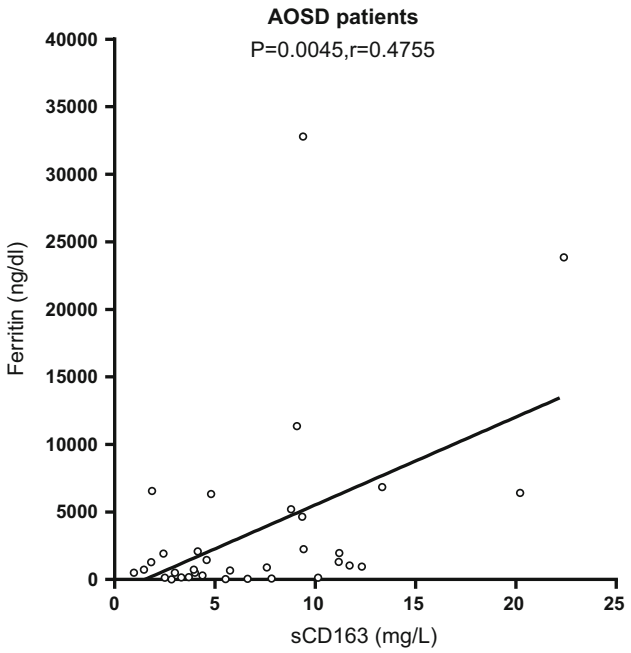

Fig. 4 Correlations between sCD163 levels and ferritin serum levels in patients with AOSD

Discussion

In our study, for the first time, sCD163 was evaluated in patients with AOSD. Even if not highly specific for the disease, sCD163 was significantly increased in active patients, representing a possible marker for disease activity. Moreover, in patients with AOSD, the sCD163 serum levels were found to positively correlate with ferritin serum levels suggesting a possible role for macrophages in ferritin production. This finding is not surprising in light of the known role of macrophages not only in the regulation of the immune response, but also in the regulation of iron homeostasis [12]. Indeed, during inflammatory conditions, the activated macrophages (named M1) are implicated in iron uptake and iron storage. On the other hand, during the resolution of inflammation, another kind of macrophage (named M2), which is more prone to tissue repair and characterized by the expression of scavenger receptors, seems to be involved in iron release. The CD163 is one of the most known M2 scavenging receptors involved in haptoglobin–hemoglobin complex uptake [12]. The sCD163 represents the serum form of this molecule released by shedding into the sera during inflammatory conditions [13]. However, although sCD163 is released into the sera during macrophage activation, its precise function has still to be defined. Different stimuli are responsible for its production including the Toll-like receptor (TLR) activation [13]; an over-expression of sCD163 has been observed in several infectious conditions [16] as well as during inflammatory diseases such as MAS [17]. Specifically, during MAS, the sCD163 levels seem to be strictly related to the degree of macrophage activation. This evidence suggested that this molecule could be used as a disease biomarker [18]. In addition, over the course of MAS, the sCD163 was already positively correlated with ferritin serum levels, suggesting a possible pathogenic relationship between these molecules. We confirmed in our study such finding, further strengthening the hypothesis of a potential macrophagic origin of ferritin along AOSD.

Ferritin can act not only as a secondary inflammatory reactant, but also as a possible serological biomarker with an active role in AOSD. Mehta et al. [19] speculated on the possible pathogenic function of ferritin in AOSD hypothesizing the presence of a mutated form with a defective iron release. Rosario et al. [4] in 2013 introduced the concept of "Hyperferritinemic syndrome" underlining the existence of a potential pathogenic role of ferritin in the above mentioned clinical conditions gathered under such common umbrella.

The biological structure of ferritin may be crucial in this process. Ferritin is composed of two different subunits, the heavy (H) and the light (L), which may assemble in a different proportion according to the tissue in which it is produced. The L subunit is usually detectable in liver or spleen tissues while the H subunit is mainly present in heart and kidney [20]. A serum isoform of ferritin, mainly composed of the L subunit, has also been recognized [20]. The H to L ratio is not fixed as it can vary in several

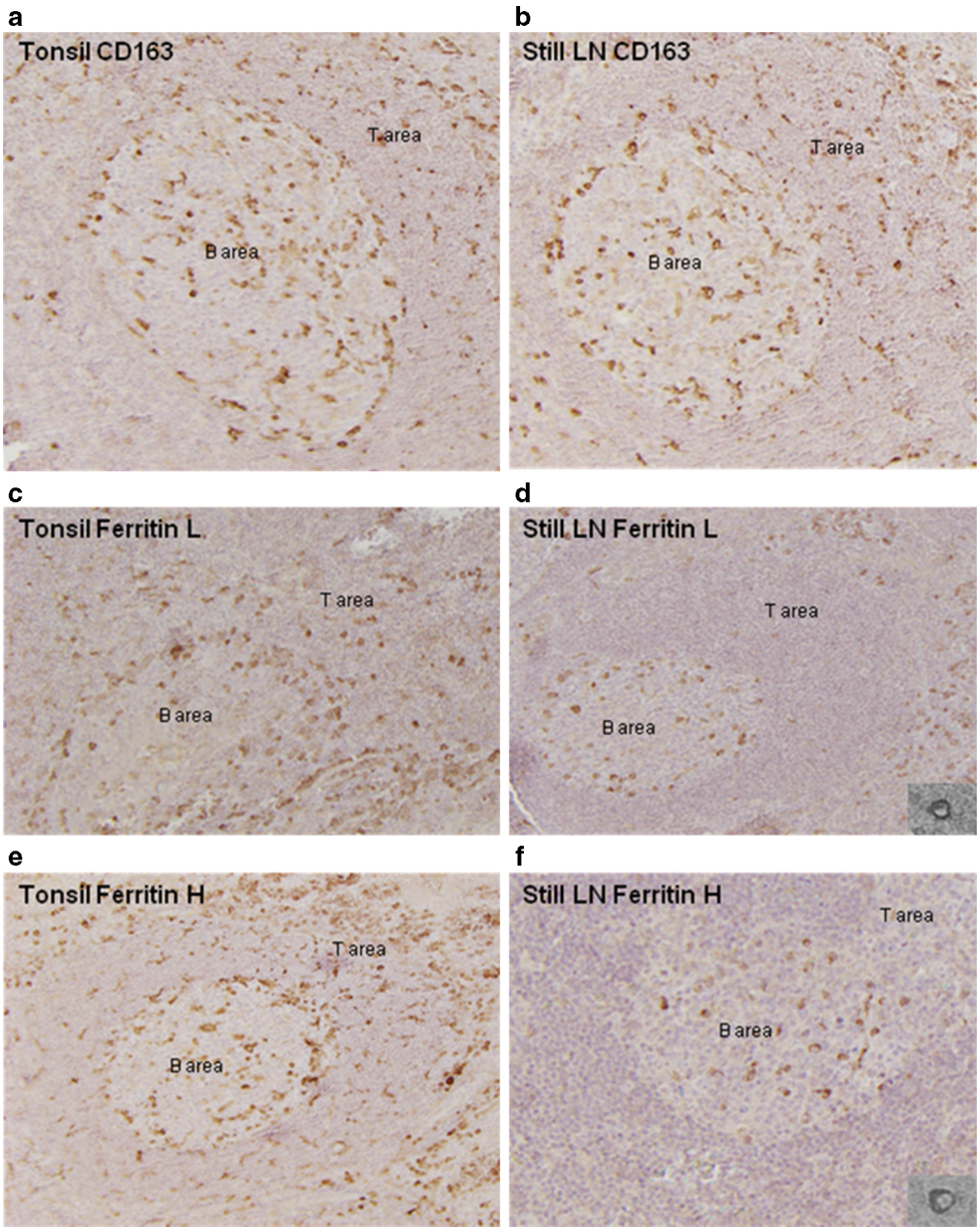

Fig. 5 CD163 is equally distributed in B and T cell areas of both lymph-node and tonsil (**a**, **b**). Ferritin subunits are highly expressed only in the B cell area of the lymph-node (**d**, **f**) while it was possible to find them widely expressed also in the T and B cell areas of the tonsil control tissue (**c**, **e**)

inflammatory and infectious conditions. An increase in the H subunit expression is driven by inflammatory stimuli [21]. Even so, the real meaning of such increased H expression during inflammatory conditions has to be defined. Recalcati et al. [22] showed that the H ferritin subunit can inhibit lymphoid and myeloid cells proliferation. Thus, ferritin may work as an immune-regulatory molecule; a specific ferritin receptor, named TIM-2, which has been identified in murine models on several immune effectors cells [23]. If we look at ferritin as a molecule implicated in the regulation of the immune response in systemic inflammatory conditions, it becomes necessary also to identify the main source of production. What we know today is that ferritin synthesis is induced by several inflammatory stimuli including cytokines secretion such as interleukin (IL)1α, IL1β, IL18, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), macrophage-colony stimulating factor (M-CSF) and IL6 [20]. For a long time, the main passive source of ferritin was supposed to be its leakage from damaged cells during inflammatory conditions.

In 2004, Ghosh et al. [24] found that ferritin L subunit is actively secreted through the classical secretory pathway rather than being released via cell lysis. The authors

demonstrated not only an active secretion of ferritin by HepG2 cells but also the existence of a serum factor capable of determining an inhibition of the production of such protein. Later on, Cohen et al. [11] reported on a significant contribution of macrophages in ferritin production in light of to the proved ability of these cells to actively secrete this protein through a non-classical secretory pathway. Despite such findings may appear to be contrasting, they both bring to evidence that ferritin is not only released into the sera from damaged cells, but can be actively secreted especially from macrophages. This active secretion may support the idea of a key role for ferritin in inflammatory conditions and especially in AOSD, in which extremely high levels of this molecule can be found.

In our work, we were able to localize the expression of CD163 also at tissue level by staining the lymph-node from a patient with AOSD. We observed macrophages expressing this specific marker diffusely distributed in the tissue. Moreover, in order to evaluate the presence of ferritin in such lymphatic tissue and to verify the eventual co-localization with CD163, a staining for H and L ferritin subunits was performed. Surprisingly, we observed a peculiar distribution of ferritin in the lymph-node from the patient. The localization of H and L subunits was not only different from CD163 but also from their distribution in the tonsil tissue from a HC. Indeed, while in the tonsil the L and the H subunits were diffusely distributed, both chains were localized only inside the B cell area of the lymph-node (Fig. 5). This is the first study demonstrating such distinct ferritin distribution in a lymphatic tissue from a patient with AOSD. According to these preliminary results, we can hypothesize a potential behavior of ferritin as an antigen in AOSD. We believe that H and L ferritin subunits may induce the activation of a specific B cell immune response and therefore contribute to the AOSD inflammatory triggering. We believe that such specific connection to B cells within lymph-nodes may induce the production of specific autoantibodies to ferritin in AOSD.

To summarize, we addressed the role of sCD163 in AOSD as a marker for disease activity, and we suggested that ferritin may have a pathogenic role, through active secretion from macrophages and through B cell activation.

Conflict of interest None declared.

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