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Original Contributions

Localization of TNF alpha in ileocolonic biopsies of patients with inflammatory bowel disease

Vincenzo Villanacci[a,*], Moris Cadei[a], Francesco Lanzarotto[b], Chiara Ricci[b], Elisabetta Antonelli[c], Rosanna Cannatelli[b], Tiziana Gulotta[a], Lucia Fontana[a], Valentina Pasquali[a], Sandra Sigala[d], Tiziana Salviato[e], Riccardo Nascimbeni[f], Gabrio Bassotti[g]

[a] Pathology Section, Department of Molecular and Translational Medicine, Spedali Civili and University of Brescia, Italy
[b] Gastroenterology Section, 1st Medical Clinic, Spedali Civili and University of Brescia, Italy
[c] Gastroenterology Unit, Perugia General Hospital, Italy
[d] Pharmacology Section, University of Brescia, Italy
[e] Department of Pathology, University of Trieste School of Medicine, Trieste, Italy
[f] Department of Surgery, University of Brescia, Italy
[g] Gastroenterology and Hepatology Section, Department of Medicine, University of Perugia School of Medicine, Perugia, Italy

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ABSTRACT

Background: Although antitumor necrosis factor alfa (TNFα) agents are widely used to treat patients with inflammatory bowel diseases (IBD) - both Crohn's disease (CD) and ulcerative colitis (UC) - there is still some uncertainty in the cell type expressing TNFα in human ileo-colonic segments.
Aims: We investigated the immunohistochemical (IHC) expression of TNFα in the ileo-colonic segments of patients with both active CD and UC, to establish its anatomic and cellular localization in the inflamed sites. Our aim was to identify patients potentially resistant to anti TNFα agents.
Patients and methods: Ileo-colonic slides of complete histological mapping of patients with CD and UC before any treatment was started were obtained, and serial sections assessed for TNFα expression, together with IHC markers for lymphocytes, macrophages, and plasma cells.
Results: TNFα was expressed in almost all inflamed segments of IBD patients, albeit with different strength, and was present, in addition to lymphocytes and, to a lesser extent, to macrophages, in plasma cells, where it had a strong positivity, as also demonstrated by colocalization of specific IHC staining. The expression of TNFα was mostly focal in CD patients and more diffuse in UC patients, likely due to the different patterns of inflammation (transmural and mucosal) of the two entities.
Conclusions: In IBD, TNFα is strongly expressed also in plasma cells, and it is easily evidenced by conventional IHC techniques. It remains to be established whether this observation might be useful in future to establish in routine biopsy samples whether patients may be responsive to treatments toward this cytokine.

1. Introduction

Antitumor necrosis factor alfa (TNFα) agents have been shown to be able to change the clinical course of inflammatory bowel diseases (IBD), and are used to treat patients with Crohn's disease (CD) and ulcerative colitis (UC) [1], even those refractory to conventional treatments [2-5]. The rationale underlying this approach is the fact that TNFα is a major pro-inflammatory cytokine playing a paramount role in the pathophysiology of IBD [6], and that blocking its inflammatory pathway leads to an effective control of the disease [7].

A recent study demonstrated that molecular imaging with fluorescent anti-TNFα antibodies might predict a therapeutic response to biological treatment in CD [8], allowing a more targeted personalized intervention. However, this study had practical limitations in that it was carried out only in inflamed areas with expensive and not widely available endoscopic techniques (i.e., confocal laser endomicroscopy), and it involved the use of fluorescent monoclonal antibodies in humans, a use currently restricted by regulatory authorities [8].

An interesting aspect is that there is still some uncertainty in the cell type expressing TNFα in human ileo-colonic segments. Although TNF is usually not detectable in normal tissues, elevated tissue levels are found in inflammatory conditions, with the main source being cells of the

* Corresponding author at: Institute of Pathology, Spedali Civili di Brescia, Piazza Spedali Civili, 1, 25100 Brescia, Italy.
E-mail address: villanac@alice.it (V. Villanacci).

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monocyte/macrophage lineage [9]. However, concerning IBD equivocal results have been reported by various authors, describing TNFα expression in macrophages [10], macrophages and CD+ cells [11], and CD14+ macrophages and CD4+ T lymphocytes [8]. Thus, the exact cellular localization of TNFα still remains an unresolved issue; in addition, this factor may also be expressed by plasma cells in conditions characterized by chronic inflammation [12].

In the present study, by a complete biopsy mapping we aimed at assessing the immunohistochemical (IHC) expression of TNFα in the ileo-colonic segments of patients with both active CD and UC, to establish its anatomic distribution and cellular localization in the inflamed sites. We feel these data could add valuable information on how to potentially individuate patients resistant to biologic therapies with anti TNFα agents and, hopefully, to individuate more targeted therapeutic approaches.

2. Patients

Patients with CD and UC were recruited in the period January 2015–January 2017 at the Gastroenterology Section of Spedali Civili and University of Brescia. Inclusion criteria were: 1) naïve patients (i.e., patients with a first diagnosis of IBD and no previous treatments) aged 18–65 yrs; 2) availability of clinical data; 3) availability of colonoscopy with ileoscopy before treatment was started; 4) complete histological mapping (at least two biopsy samples taken from each anatomical site from the terminal ileum to the rectum, correctly oriented on acetate cellulose filters). Biopsy samples from these patients were retrieved from the archive of Pathology of Spedali Civili and University of Brescia and processed as described below.

3. Methods

Serial sections from each anatomical segments were obtained from all patients; the first was stained with H&E and the subsequent ones processed for IHC evaluation (see below).

Staining for IHC was carried out automatically by means of Leica Bond Max™ technology. Firstly, sections were stained with CD3 (Thermo Fisher Scientific, Waltham, MA, USA, dilution 1:100), a pan-T lymphocyte marker, and CD68 (Leica Microsystems, Buccinasco, Italy, dilution 1:400), to evaluate macrophages. Samples were incubated with EDTA for 15 min to achieve antigen retrieval. Then, a mouse anti-TNFα antibody (Thermo Fisher Scientific, working dilution 1:50) was used as primary antibody, while the staining was obtained through a DAB-based revelation system. Moreover, antibodies toward κ and λ chains (kappa and lambda probes, Leica Microsystems) were tested to confirm the presence and distribution of plasma cells. To further confirm the presence of TNFα in plasma cells, additional sections were assessed for co-localization by means of a double immunostaining for plasma cells with MUM1 (Dako, Santa Clara, CA, USA, dilution 1:80), since the anti-TNFα antibody stains the cytoplasm and MUM1 the nuclei.

4. Data analysis

Patients with IBD were phenotypically defined according to the Montreal classification [13]. The histological diagnosis and the disease activity were formulated on H&E sections by an experienced pathologist, according to a standard method [14], and judged to be moderate to severe in all patients.

The number of plasma cells (MUM1 positive), macrophages (CD68 positive) and lymphocytes (CD3 positive) for each segment (and expressed as median [95%CI]) was calculated by counting five optical fields at 40× for each of the two biopsy samples obtained in that segment. To establish the localization of anti-TNFα positive cells, sequential sections stained with H&E, CD3, CD68, κ and λ chains, and anti-TNFα were assessed at 40× magnification and the percentage of TNFα was calculated.

Table 1
Demographic and phenotypic (according to Montreal classification) features of IBD patients under investigation.

	UC	CD
Gender (male/female)	12/5	10/8
Age, yrs (mean, range)	40 (24–75)	39 (19–69)
Disease localization (number)	Proctitis (3)	Ileal (5)
	Left-sided (11)	Colonic (1)
	Pancolitis (3)	Ileo-colonic (12)
Disease activity	Moderate (15)	non-stricturing, non-penetrating (11)
	Severe (2)	stricturing (5)
		Penetrating (2)

Table 2
Median [95%CI] number of PC, M, and L in the different segments of CD patients; the parentheses show the percentage of TNFα positive cells in these cell populations.

	I	C-A	T	D	S	R
PC	21.5 [19–25] (77)	21.5 [18.4–26.4] (81)	21 [16–24] (77)	21.5 [16–24] (75)	19 [15–23] (80)	17 [14–21] (75)
M	10.5 [7–13.4] (73)	11.5 [8.4–16] (74)	12 [8.4–18] (75)	14 [10.4–17] (78)	12 [7.4–17] (78)	7 [5.4–18] (78)
L	41* [38–48] (84)	29 [25–39] (87)	33 [29–39.5] (82)	37 [26–45] (82)	27 [19.5–38] (81)	25 [21.5–33.7] (77)

Abbreviations: I = ileum; C-A = cecum-ascending; T = transverse; D = descending; S = sigmoid; R = rectum; PC = plasma cells; M = macrophages; L = lymphocytes.
* $p = 0.003$ vs R.

Table 3
Median [95%CI] number of PC, M, and L in the different segments of UC patients; the parentheses show the percentage of TNFα positive cells in these cell populations.

	I	C-A	T	D	S	R
PC	23 [17–29] (87)	25 [18.5–43] (86)	26 [20–41] (85)	24.5 [19–43] (86)	42 [35–48] (87)	43 [42–49] (86)
M	6 [2–13] (79)	7 [5–10.5] (78)	9* [4.5–12] (82)	7.5 [6–9] (77)	10 [7–14] (84)	12 [8–15] (84)
L	31.5 [15–48] (82)	33 [25.5–47] (85)	39 [28.5–53] (75)	37 [32–51] (85)	49 [42–50] (85)	51 [47–54] (81)

Abbreviations: I = ileum; C-A = cecum-ascending; T = transverse; D = descending; S = sigmoid; R = rectum; PC = plasma cells; M = macrophages; L = lymphocytes.
* $p = 0.018$ vs R.

Concerning IHC quantification of TNFα positivity, a determined anatomical segment was considered as positive when examination of at least five optical fields for each of the two biopsy samples obtained in that segment constantly yielded an arbitrary cut-off value of 30 or more stained cells per microscopic field at a magnification of 40. This arbitrary cut-off was based on that proposed by Atreya et al. [8], but we set a higher value for strong positivity (30 cells/field instead of 20). However, depending on whether CD or UC patients were examined, this positivity could be focal (especially CD) or diffuse (especially UC).

5. Statistical analysis

Differences in cell numbers and the percentages between the various anatomical segments for each cell type investigated were assessed by

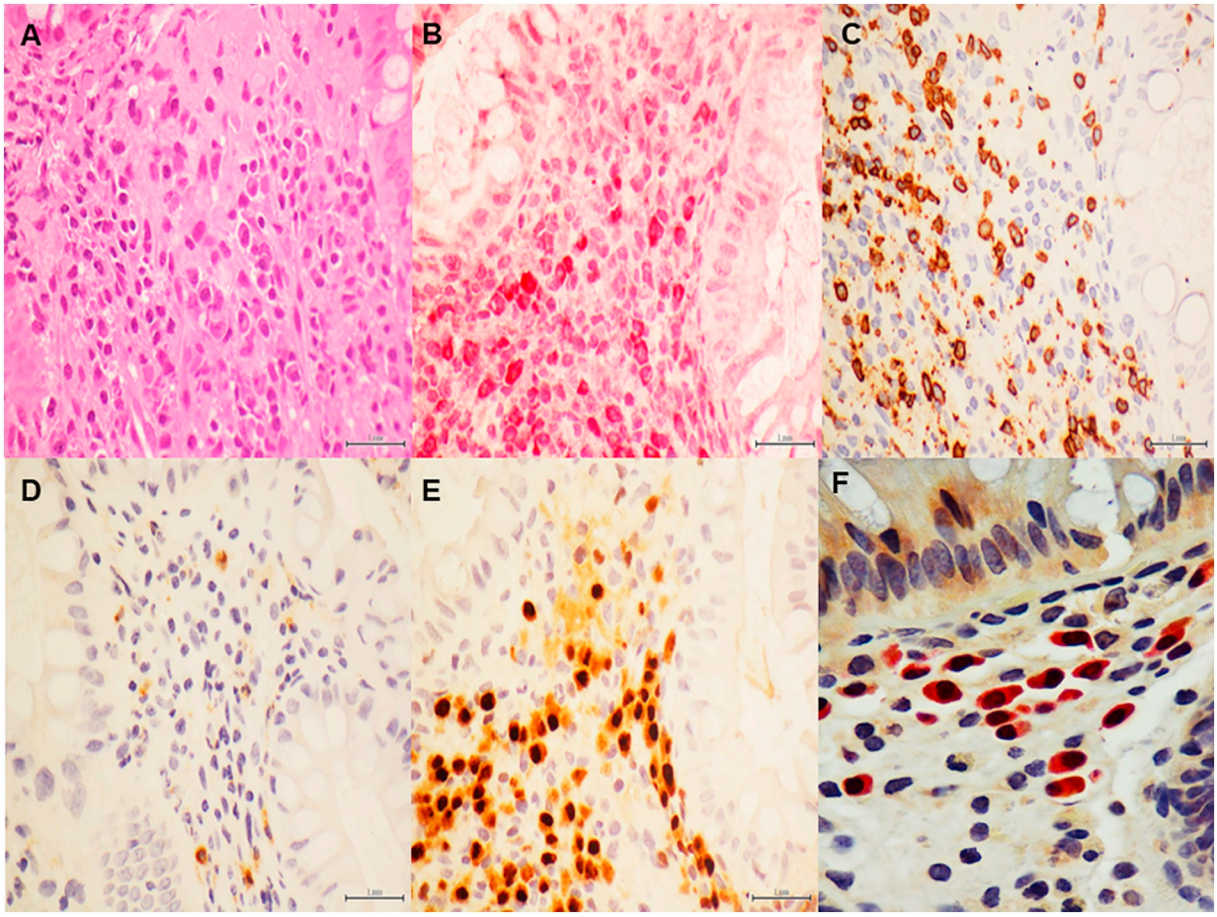

Fig. 1. Sequential sections of a UC patient showing the conventional H&E staining (A) and the positivity for TNF (B), CD3 (C), CD68 (D), MUM1 (E), and double immunostain for MUM1 (brown color in the nuclei) and TNFα (red color in the cytoplasm) (F). Original magnification ×40. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

means of the two-tailed analysis of variance (ANOVA) for repeated measures, with Bonferroni correction. Values of $p < 0.05$ were chosen to reject the null hypothesis.

6. Ethical considerations

Ethical approval for the study was granted by the IRB of Spedali Civili and University of Brescia; informed consent was obtained from all patients.

7. Results

Table 1 shows demographic and phenotypic features of the IBD patients included in the study (18 patients with CD and 17 with UC). Tables 2 and 3 show the median number of each cell type in the various anatomical segments for both CD and UC patients, together with the percentage of TNFα positive cells within each group. Statistical analysis did not show significant differences in the number of each cell type between the various anatomical segments of CD and UC patients, except for the higher number of ileal vs rectal lymphocytes ($p = 0.003$) in CD patients and the lower number of monocytes in the transverse colon vs rectum ($p = 0.018$) in UC patients. The morphological analysis of sequential colonic histological sections showed that TNFα positivity was found within plasma cells, lymphocytes and, to a lesser extent, macrophages (Figs. 1 and 2). Concerning plasma cells, this observation was also strengthened by IHC results obtained with the co-localization of the plasma cell biomarker MUM1 with TNFα (Fig. 1). No statistical differences were however found in the percentage of TNFα positive cells for each cell subtype in the various anatomical segments, although there was a trend toward a lesser expression in macrophages (Tables 2 and 3).

Immunohistochemical quantification is summarized in Table 4. As expected, there was a strong association between the grade of inflammation and the TNFα positivity. All CD patients displayed a focal positivity in the inflamed anatomic segments, whereas a diffuse positivity was found in at least one anatomical segment in 9/18 (50%) patients. In 7 of these patients the diffuse positivity was present in more than one segment. All UC patients also displayed focal positivity in the inflamed anatomic segments (Fig. 3 A); a diffuse positivity (Fig. 3 B) was present in at least one anatomical segment in 16/17 (94%) patients. This positivity had a more homogeneous anatomical distribution, and it was concentrated in the distal, more inflamed segments, in 14 of these patients.

8. Discussion

Although TNFα represents a key factor in the pathogenesis of IBD, about 30% of patients display a primary non-response (i.e., they do not show improvement after induction therapy), and a further 20%/year show a secondary non-response (i.e., loss of response in the time course) [15-18].

On this basis, the identification of predictive biomarkers to establish whether a patient will be responding to anti-TNFα agents is intuitive and is still an unresolved need. In fact, a biomarker of TNFα sensitivity would avoid unnecessary exposure of the patient to the drug and to its side effects, that sometimes may be serious [19,20], while reducing

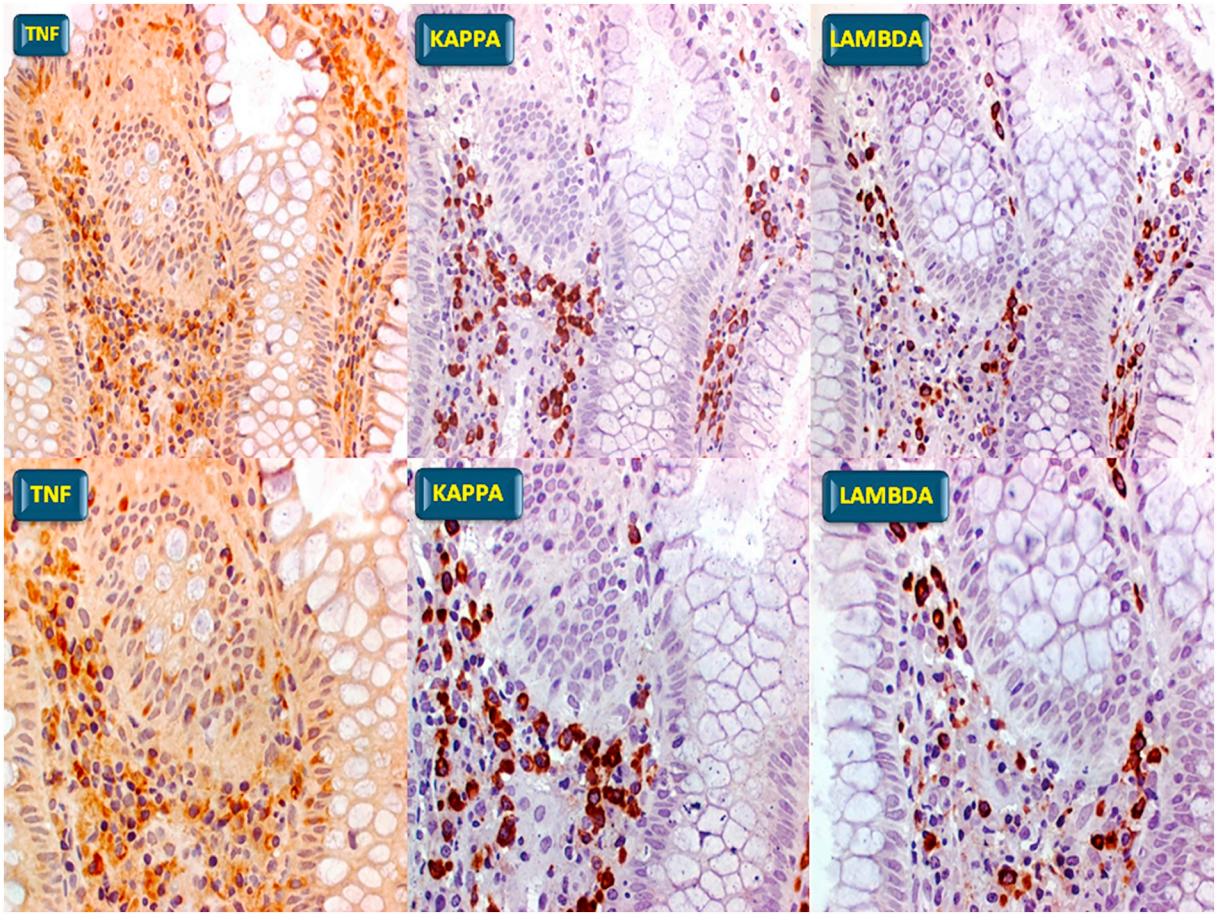

Fig. 2. Comparison between TNFα and kappa-lambda immunostains reveals the cellular expression of TNFα in plasma cells. Original magnification, ×40.

Table 4
TNFα positivity in ulcerative colitis (UC) and Crohn's disease (CD).

	UC no activity	UC < 30	UC > 30	CD < 30	CD > 30
I	13	4	0	15	3
C-A	10	5	2	13	5
T	7	6	4	13	5
D	3	8	6	13	5
S	0	4	13	16	2
R	0	1	16	18	0

< 30 = less than 30 positive cells.
> 30 = more than 30 positive cells.
Abbreviations: I = ileum; C-A = cecum-ascending; T = transverse; D = descending; S = sigmoid; R = rectum.

spending as well. Unfortunately, apart from the demonstration that patients with high blood levels of C reactive protein respond better to anti-TNFα agents [21], no other biomarkers are available in the daily routine. The proposed apoptotic index to predict response to infliximab [22], although interesting, seems relatively complex and must be still validated in large prospective trials. Thus, the identification of patients that will respond to anti-TNFα agents remains a major clinical challenge. In addition, there is the need to better understand the role of TNFα in these conditions, especially at the inflamed intestinal segments level, to establish why different therapeutic responses are observed in different subjects. Unfortunately, studies addressing this issue are scarce. A previous study in UC patients employing IHC techniques showed that TNFα expression correlates to the degree of colonic inflammation [11], and that tissue levels of TNFα may predict response to infliximab treatment [23]. A more recent study in patients with CD suggests that the high expression of TNFα in the inflamed ileo-colic tissues of the patients might predict the response to biological agents [8]. However, these studies were carried out with techniques scarcely applicable to real-life conditions, and thereby limited to an experimental setting.

Our results, obtained by means of conventional techniques easy available in most Pathology Units, show that in actively inflamed, moderate to severe IBD (both CD and UC) patients, TNFα positivity was constantly and selectively detected in the mucosa of the affected segments, although with a different intensity of expression. This, once again, stresses the importance of this cytokine in the pathogenesis of these entities [6] and it remarks its important role as a therapeutic target [7]. However, although the mucosal samples were obtained in patients' naïve from any therapy, we observed that CD patients displayed a quite heterogeneous pattern of TNFα expression in the inflamed areas, with a prevalence of focal expression, whereas diffuse expression was more consistent and homogeneous in UC patients, especially in the distal colonic segments. This observation likely reflects the pathophysiologic differences between the two conditions, CD displaying a patchy and transmural inflammatory state, while a predominant mucosal and continuous inflammation can be detected in UC [24]. Concerning the cellular localization of TNFα, conflicting results are present in literature [9-12]. In this study, we demonstrated that TNFα positivity, in addition to be present in lymphocytes and, to a lesser extent, in macrophages, was also strongly and diffusely localized within plasma cells. This positivity was documented by the use of κ and λ probes and, especially by the co-localization of MUM1, a marker mostly expressed in plasma cells and in 1–5% of T lymphocytes [25]. We would like to underline this point, since previous studies (carried out without the use of serial sections or double antibody co-localization) have yielded equivocal results, describing TNFα expression in

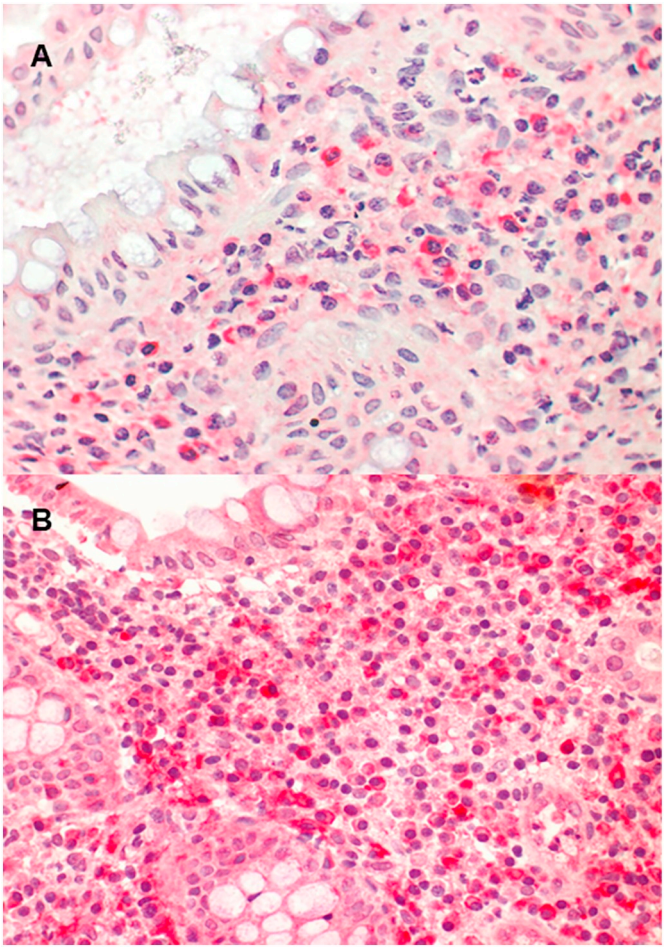

Fig. 3. Different expression of TNFα: A, focal positivity; B, diffuse positivity. Original magnification: ×40.

macrophages [10], macrophages and CD+ cells [11,26], and CD14+ macrophages and CD4+ T lymphocytes [8].

To the best of our knowledge, plasma cells were never investigated before in this setting. Since TNFα may also be expressed by plasma cells in pathological conditions characterized by a chronic inflammation [12] our results might also have therapeutic implications, suggesting a new cellular target to be potentially addressed. Indeed, plasma cells in IBD likely play some important role, as shown by their precocious presence from the very early phases of these diseases [27,28]. In addition, these findings could explain the fact of the scarce efficacy of rituximab (anti CD20) in UC [29], as CD20 is downregulated during plasma cell differentiation [30].

Finally, extrapolating these observations to a clinical context, we feel that the results presented here might also have some practical implications. In fact, since colonoscopy with biopsy sampling has a pivotal role in the diagnosis and management of IBD patients [31], assessing the expression of TNFα in endoscopic biopsies at first diagnosis, together with a precise histological assessment, could potentially identify patients responding to anti-TNFα agents. This has been recently shown by Atreya and coworkers for CD patients [8]. Of course, due to the more frequent colonic localization of the inflammatory activity in UC, particularly in the distal segments, this approach could perhaps be more useful in these patients, as shown by preliminary data from our institutions (Villanacci V, Bassotti G, unpublished data). Thus, in theory it could be possible to avoid unnecessary therapeutic delays, and perhaps unnecessary costs, in IBD patients needing health benefits. Such hypothesis needs to be clinically tested, even because there is still no validated standardization of TNFα positivity, and studies addressing these issues are under way, with the hope to offer a more targeted approach to these disabling conditions.

Conflict of interest statement

None declared.

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