Pathology International

Pathology International 2019; **00:** 1–12 doi:10.1111/pin.12794

Original Article

Prediction of steroid demand in the treatment of patients with ulcerative colitis by immunohistochemical analysis of the mucosal microenvironment and immune checkpoint: role of macrophages and regulatory markers in disease severity

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We aimed to characterize the mucosal immune microenvironment and immune checkpoint of Ulcerative colitis (UC) by immunohistochemistry with correlation to prognosis: requirement of second-line steroid-therapy within the 2-years after diagnosis (SR). A series of 72 cases included 56 UC, 43 non-SR (with first-line treatment 5-ASA) and 13 SR, 11 infectious colitis and 5 normal colonic biopsies. Normal mucosa was characterized by low infiltrates but high BTLA and TNFRSF14. Compared to normal, UC had increased pan-immune-markers of CD3, CD8, FOXP3, PD-1, CD68, CD16, CD163, PTX3 and CD11C but had decreased BTLA ($P < 0.05$); by GSEA analysis comparable results were found in an independent UC gene-expression-data set (GSE38713). Compared to infectious, UC had higher CD4, CD8, PTX3 and CD11C but lower BTLA ($P < 0.05$). Compared to non-SR, SR had lower FOXP3 + Tregs (Odds-Ratio = 0.114, $P = 0.002$), PD-1 (OR = 0.176, $P = 0.002$) and CD163/CD68 M2-ratio (OR, 0.019, $P = 0.019$) but higher CD68 + pan-macrophages (OR = 6.034, $P = 0.002$). Higher Baron endoscopic and Geboes histologic disease activity scores also correlated with SR. In summary, UC was characterized by increased pan-immune-markers, normal TNFRSF14 and low BTLA. SR had increased CD68 + pan-macrophages but lower immune inhibitors of FOXP3 + Tregs, PD-1 and CD163/CD68 M2-macrophage ratio. In conclusion, alterations of the immune homeostasis mechanisms are relevant in the UC pathogenesis and steroid-requiring situation.

Key words: BTLA, CD163, FOXP3, GSEA, immune homeostasis, microenvironment biomarkers and immune checkpoint, macrophages, PD-1, prognosis, TNFRSF14, Ulcerative colitis

INTRODUCTION

In Japan, the prevalence of UC cases is approximately 160,000 (around 0.13% of the total population) and the incidence has been recently increasing by nearly 10,000 patients a year.[1] Although the cause of UC is still unknown, more than 50 susceptibility loci and disease-associated genes have been identified.[2] UC is a multifactorial disease that is triggered by several factors such as genetic predisposition, enteric bacteria, and diet. One of the major pathological conditions leading to UC is thought to be overactive immunoreactions due to a defective immune regulation (ie immune homeostasis and tolerance).[3] UC affects mainly mucosa of rectum and colon. The initial treatment is 5-aminosalicylic acid (5-ASA, mesalamine)[4] and when there is no improvement or exacerbation, steroids are used as the first choice, followed by immunomodulators and biopharmaceuticals.[5] Therefore, steroid

Author Contributions: ST, JC, YYK, HN, MD, JI, KT, JN, YT, TU, MM, GRD, TS, NN and TM made substantial contributions to the conception or design of the work; the acquisition, analysis, and interpretation of data for the work. ST and JC made analysis and draft the manuscript. JC, MM and NN made the final approval of the version to be published.

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Received 22 November 2018. Revised 7 February 2019. Accepted for publication 24 February 2019.

requirement (SR) can be a simple and easy marker for the severity or activity of the disease. A major tool to diagnose UC is colonoscopy combined with biopsy; this study aimed to investigate histological markers of the microenvironment and immune checkpoint at diagnosis using the colonoscopy formalin-fixed paraffin-embedded tissue (FFPET) samples that are available for diagnosis and research. We aimed to determine whether the prognosis would be more severe, or the steroid therapy would be required, in other words.

A defective mucosal immune regulation may lead to the development of inflammatory bowel disease (IBD) including UC[6] as the activation of the immune system will eventually lead to the destruction of the epithelial layer. Some of the mechanisms have been postulated as commented in the following sentences. In this project we used biomarkers, immunohistochemical markers, to identify cell types and immune pathways present in the intestinal mucosa as detailed in the Table 1. We identified T lymphocytes (CD3), T helper cells (CD4, UC is characterized by an imbalance of CD4 + T cells),[7] cytotoxic T lymphocytes (CD8, also present in active stages of UC), NK cells (CD56, involved in UC pathogenesis as immune regulatory and tissue repair cells),[8] Tregs (FOXP3, immune response suppressor, FOXP3 mutation leads to Immune dysregulation, polyendocrinopathy, enteropathy, X-linked *IPEX syndrome* which hallmark is autoimmune enteropathy, including colitis),[9] PD-1 and BTLA receptors (co-inhibitory signaling pathways) and its ligand TNFRSF14 (Pro-inflammatory, its signaling promotes colitis),[10,11] pan-macrophages (CD68), Pro-inflammatory M1-like (CD16), anti-inflammatory M2-like macrophages [CD163 and pentraxin 3 (PTX3); M2-like macrophages protect against experimental colitis][12] and immune regulatory DCs (CD11C, also positive in M1-like macrophages; CD11C + myeloid cells are involved in the intestinal immune homeostasis).[13]

In this project we performed a comprehensive immunohistochemical analysis of the mucosa, including some markers that to our knowledge have not been explored in UC yet such as PD-1, PTX3, BTLA and TNFRSF14 to search for prognostic markers. Our results showed that UC requiring steroid therapy (steroid-requiring, SR) had increased expression of CD68 and decreased FOXP3, PD-1 and anti-inflammatory M2-like macrophages (as expressed by the CD163/CD68 ratio).

MATERIALS AND METHODS

Subjects of study, steroids requiring (SR) criteria and tissue samples

The series was comprised of 72 cases including 56 UC, 11 infectious colitis as "positive" control and 5 normal colonic mucosa as a "negative" control. The samples were retrospectively selected from Japanese patients from 2005 to 2013 and retrieved from the Department of Pathology, Tokai University, School of Medicine. The selection criteria were biopsies taken in the colonoscopies at diagnosis and the presence of adequate tissue area for histological evaluation. When multiple biopsies were present, the most inflamed one was selected. The location of the biopsies that were used for the immunohistochemical procedures was predominantly from the left side of the colon (60 of 72, 83%) while the right and transverse side were less frequent (10% and 7%, respectively). Left-sided biopsies were descending colon 22% (13 of 60), sigma 18% (11 of 60) and rectum 60% (36 of 60). The gender was male in 65% (47 of 72) and the mean age was 42 years old. Of note, control biopsies were also mainly left-sided. In the infectious colitis control group, ischemic cases were not present. Under histological evaluation the normal mucosa was not depleted of inflammatory infiltrate.

The clinical disease activity was evaluated basically by the Ulcerative Colitis Disease Activity Index (UC-DAI).[14] Initially all the patients had started treatment with oral 5-ASA (mesalazine) preparations with or without probiotics. When the initial treatment failed to induct the patients to a remission

Table 1 Primary Antibodies for immunohistochemistry

Marker	Target	Clone	Company
CD3 epsilon	T cell lineage (T lymphocytes)	LN10	Novocastra
CD4	T helper cells (Th) (also macrophages)	4B12	Novocastra
CD8 alfa	Cytotoxic T lymphocytes (Tc)	4B11	Novocastra
CD56 (NCAM1)	Natural Killer cells (NK)	CD564	Novocastra
FOXP3	Regulatory T lymphocytes (Tregs)	236 A	CNIO
PD-1 (PDCD1)	Co-inhibitory and FTH cells	NAT105	CNIO
BTLA	Co-inhibitory and FTH cells	FLO67B	CNIO
TNFRSF14 (HVEM)	Costimulatory	Polyclonal	Abcam
CD68	Pan macrophages	514H12	Novocastra
CD16 (FCGR3A)	M1-like Pro-inflammatory macrophages	2H7	Novocastra
CD163	M2-like alternatively activated macrophages	10D6	Novocastra
PTX3	M2-like alternatively activated macrophages	PPZ1228	Perseus Proteomics
CD11C (ITGAX)	Immune regulatory dendritic cells (also M1-like macrophages)	3D11	Novocastra

CNIO, Spanish National Cancer Research Centre; FTH cells, follicular T helper cells. Of note, PTX3 and CD16 can be expressed by other cell populations such an endothelial cells and NK cells, respectively. CD4 can also be found in antigen presenting cells (eg macrophages).

state (UC-DAI score, 1–2) or the disease relapsed defined by the symptoms (UC-DAI > 2, with bloody stool) as well as by laboratory data and/or colonoscopy, more aggressive treatments were needed to control UC (prednisolone as the first choice). During a 2-year follow-up after the initial diagnosis of UC, 13 patients needed steroid therapy (UC requiring oral steroids, ie SR) while 43 patients did not require the use of steroids (ie disease kept in remission without flares, 5-ASA responsive and without the need of using of oral steroids, non-SR). Of note, the differentiation of these two groups of patients follows the European Crohn's and Colitis Organisation (ECCO), left-sided colitis treatment algorithms (http://www.e-guide.ecco-ibd.eu/algorithm/left-sided-colitis).

This study was approved by the Institutional Review Board of clinical research (13R-119) and was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Endoscopic assessment

The assessment of disease activity, extend and behavior, was evaluated using the Baron Score [15], that ranges from 0 (normal mucosa), 1 (mild), 2 (moderate) and 3 (severe) (Supplementary Table 2). Endoscopic remission was defined as a Baron Score of 0 to 1. Endoscopic images taken during the colonoscopy at the biopsy time were used for the assessment.

Histologic disease activity assessment

Histological assessment at diagnosis was based on the Geboes Score,[15] that it is still a valid method,[16,17] using the hematoxylin and eosin stainings. The score is detailed at the Supplementary Table 3.

Immunohistochemistry and marker quantification

Table 1 describes the antibodies that were used and their target population. Immunohistochemistry (IHC) staining was performed in an automated system according to the manufacturer's instructions [Leica Bond-Max and Bond Polymer Refine Detection, DS9800. Novocastra (NV), Leica Microsystems K.K., Tokyo, Japan].[18,19] In summary, the staining process consisted on dewax, hydration, antigen retrieval, primary antibody, peroxidase block, postprimary reagent, HRP-polymer, DAB and hematoxylin steps. Antigen retrieval consisted on Bond ER2 solution for 20 minutes in all the antibodies except for TNFRSF14, CD16 and PTX3 that used Bond ER1 solution. The incubation time of the primary antibodies was the standard 15 minutes in most of the cases.

The IHC slides were examined in a brightfield microscope by two of observers (Tsuda S. and Carreras J., BX53 and BX63 series, DP73 digital camera, cellSens software; Olympus K.K., Tokyo, Japan). For each marker the distribution and percentage were assessed following a strategy as previously described[18,19] and reflects the percentage of positivity of the marker within the immune inflammatory infiltrate in the lamina propria of the mucosa. The markers were recorded as an ordinal variable [0 (<5%), + 1 (5–25%), + 2 (25–50%) and + 3 (>50%)] (Fig. 1). Due to the low level of expression, FOXP3 and PTX3 were classified as 0 (<1%), + 1 (1–5%), + 2 (5–25%) and + 3 (>25%) (Figure 1a). All markers had a membranous/cytoplasmic distribution with exception of FOXP3 that was nuclear. All markers had previously been tested for IHC optimization and titration on reactive tonsils (positive external control). The original magnification of the figures was 200 × (Olympus BX63 series). The composition of the figures for manuscript was performed using GNU Image Manipulation software (GIMP 2.8.18).

Gene expression analysis

Gene expression data from active ulcerous colitis (n = 15) and noninflammatory control (n = 13) was obtained from the Gene Expression Omnibus database (GSE38713, Affymetrix Human Genome U133 Plus 2.0 Array, processed data).[20] Gene Set Enrichment Analysis (GSEA), which included the use of the Molecular Signatures Database (MSigDB), was performed to determine whether a priori defined set of genes showed statistically significant, concordant differences between active UC and control.[21,22] The set of genes corresponding to our immunohistochemical markers had been refined using the protein-protein interaction network application of STRING version 10.5.[23]

Statistical analysis

SPSS software was used for the data analysis (IBM® SPSS® Statistics Version 25, IBM, New York, United States). Comparisons between groups and variables was made with crosstabulation and Pearson Chi-Square tests. Continuity correction, likelihood ratio, Fisher's exact and linear-by-linear association tests were also considered when necessary. Nonparametric tests for independent samples either the Mann-Whitney U test (2 samples) or Kruskal-Wallis 1-way ANOVA (k samples) were used to automatically compare distributions across groups as well. Binary logistic regression both univariate and multivariate were used to differentiate between non-SR versus SR UC and calculate the odds ratio (OR). Unsupervised classification was made by means of hierarchical cluster analysis. The significance level was set up a priori at 0.05.

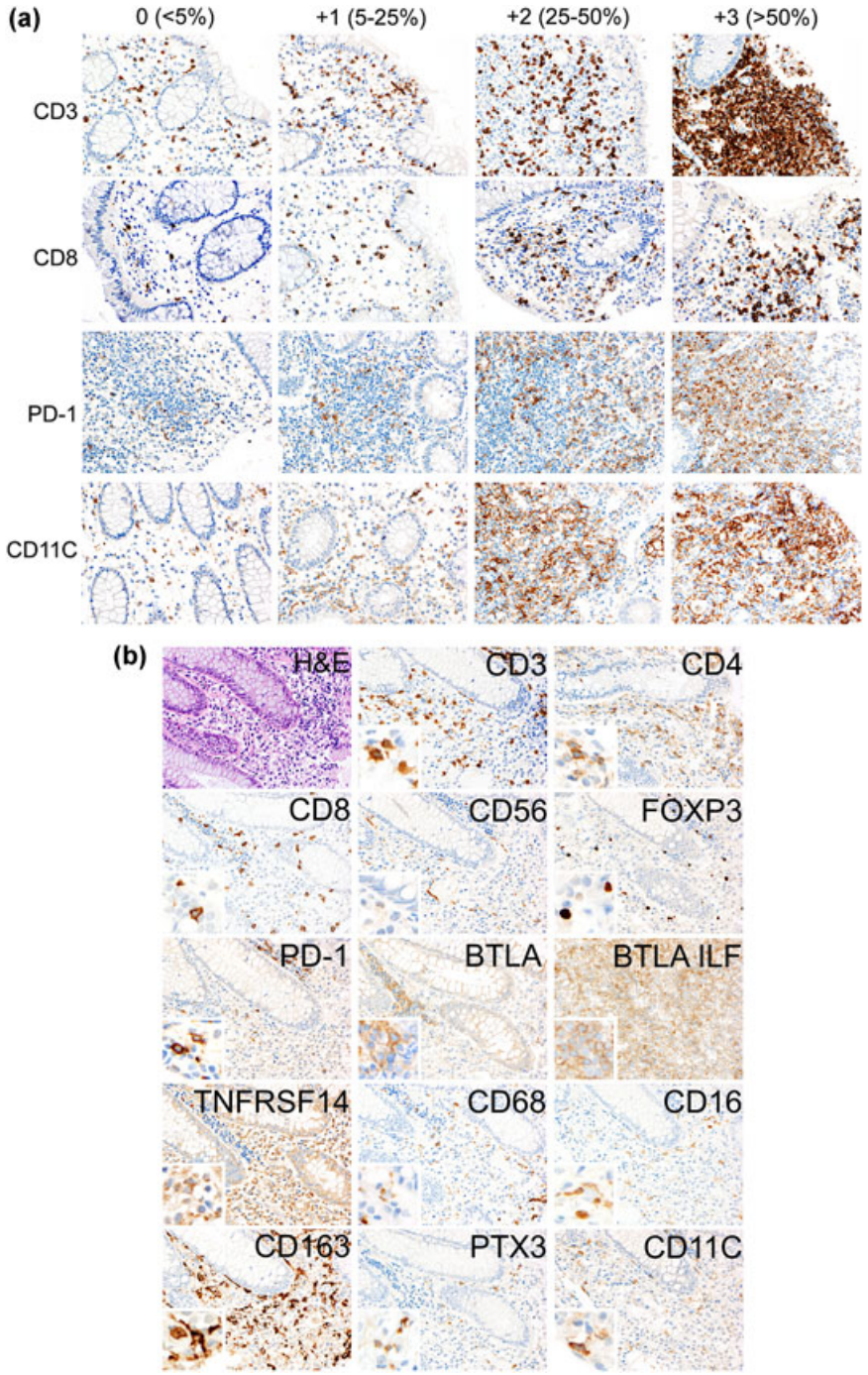


Figure 1 **(a)**. Assessment of the immune markers by immunohistochemistry. Each marker was quantified based on the percentage of expression in the inflammatory infiltrate of the lamina propria of the mucosa as follows: 0 (<5%), + 1 (5–25%), + 2 (25–50%) and + 3 (>50%). This figure shows the distribution of CD3, CD8, PD-1 and CD11C that identify T-lymphocytes, cytotoxic T-lymphocytes, PD-1 receptor and immune regulatory dendritic cells, respectively. The figures are from normal and UC cases, original magnification 200 ×. **(b).** Expression of the immune markers in ulcerative colitis (UC)**.** This figure shows the distribution of the different immune markers in a characteristic case of non-steroid requiring (non-SR) UC (case id.50). In general, UC was characterized by increased expression of both Pro-inflammatory and anti-inflammatory markers but low BTLA. ILF: isolated lymphoid follicle. CD3, + 2; CD4, + 2; CD8, + 2; CD56, + 1; FOXP3, + 2; PD-1, + 2; BTLA, + 3; BTLA ILF, + 2; TNFRSF14, + 3; CD68, + 1; CD16, + 2; CD163, + 2; PTX3, + 2; and CD11C, + 2. Original magnification 200 ×.

RESULTS

Correlation between endoscopic and histological assessments of mucosal inflammation

The frequencies according to the endoscopic Baron and the histological Geboes Scores at diagnosis are shown in Table 2. Higher scores correlated with steroids-requiring (SR). The Baron Score showed higher association [Odds Ratio (OR) of 7.8, $P = 0.007$] than the Geboes Score (OR of 2.5, $P = 0.01$).

Correlation of the immune markers with normal, infectious and ulcerative colitis

The detailed data is shown in the Supplementary tables 1.A., 1.B and 1.C. A typical case of non-SR UC (case id.50) is shown in Figure 1b. The differences are also clearly visualized in the radar-type plot of Figure 2a,b.

Normal colonic mucosa was characterized by low frequencies of all immune markers with exception of BTLA (60%, + 3) and TNFRSF14 (60%, + 3). In comparison,

Table 2 Correlation endoscopic Baron and histologic Geboes Scores at diagnosis with Ulcerative Colitis

	non-SR		Steroids-requiring (SR)			Total	
	No.	(%)	No.	(%)	*P* value	No.	%
Baron Score							
0	0	(0)	0	(0)		0	(0)
1	22	(51.2)	2	(15.4)		24	(42.9)
2	21	(48.8)	8	(61.5)		29	(51.8)
3	0	(0)	3	(23.1)	0.001	3	(5.4)
Geboes Score							
0	0	(0)	0	(0)		0	(0)
1	5	(11.6)	0	(0)		5	(8.9)
2	23	(53.5)	4	(30.8)		27	(48.2)
3	10	(23.3)	3	(23.1)		13	(23.2)
4	4	(9.3)	5	(38.5)		9	(16.1)
5	1	(2.3)	1	(7.2)	0.07	2	(3.6)

UC, ulcerative colitis. SR, requiring steroid therapy. Binary logistic regression, UC subtype (depending variable) according to Baron Score, OR, 7.8 ($P = 0.007$); according to Geboes Score, OR, 2.5 ($P = 0.010$).

infectious colitis was high in most of the markers including CD3 (72.7%, + 2 and + 3), CD8 (90.9%, + 2 and + 3), FOXP3 (63.6%, + 2 and + 3), PD-1 (63.7%, + 2 and + 3), CD68 (100%, + 1 and + 2), CD163 (72.8%, + 2) and PTX3 (63.6%, + 1) (all markers $P < 0.05$).

In a similarly way, in comparison to normal mucosa UC was also characterized by an increase of most of the immune markers, including CD3 (73.2%, + 2 and + 3), CD8 (78.5%, + 2 and + 3), FOXP3 (69.6%, + 2 and + 3), PD-1 (46.4%, + 2 and + 3), CD68 (35.8%, + 2 and + 3), CD16 (26.8%, + 2 and + 3), CD163 (75%, + 2 and + 3), PTX3 (44.6%, + 2) and CD11C (80.4%, + 2 and + 3) (all markers $P < 0.05$) but low BTLA [BTLA + 3, normal vs UC: 60% vs 5.4% ($P = 0.001$)].

In comparison to infectious colitis, UC was characterized by higher CD4 (60.7%, + 2 and + 3, $P = 0.028$), CD8 (78.5%, + 2 and + 3, OR, 12.89), PTX3 (44.6%, + 2, OR, 3.6) and CD11C (80.4%, + 2 and + 3, OR, 28.1) (all markers $P < 0.05$) but lower BTLA [BTLA + 3, infectious vs UC: 72.7% vs 5.4% ($P = 8.5386E\text{-}7$), OR, 0.091].

In summary, normal mucosa was characterized by a low inflammatory infiltrate but high BTLA and TNFRSF14. The presence of infectious colitis and UC resulted in an increase of most of the immune markers, an increment that was more significant in case of UC. In comparison to infectious colitis, UC had higher CD8, PTX3, CD11C. Both normal mucosa and infectious colitis had high BTLA. Conversely, lower BTLA was characteristic of UC.

Correlation between the immune markers and SR outcome in ulcerative colitis

The detailed data is present in Tables 3 and 4 as well as the Supplementary tables 3.1, 3.2 and 3.3. The comparison between non-SR (ie 5-ASA-responsive, case id.55) vs SR (steroids-requiring UC, case id.62) is shown in Figure 2a.

Positive correlation was found for FOXP3, PD-1 and CD68 as follows: SR was characterized by lower numbers FOXP3 + Tregs ($P = 0.002$), lower PD-1 expression ($P = 0.004$) and higher CD68 + pan-macrophages ($P = 0.004$). SR vs non-SR, high FOXP3 (+2 and +3), 30.8% vs. 81.4%; high PD-1 (+2 and +3), 7.7% vs 58.1%; high CD68 (+2 and +3), 76.9% vs. 23.2%, respectively. Interestingly, SR had lower alternatively activated M2-like macrophages as expressed by the CD163/CD68 ratio. High CD163/CD68 ratios (+2 and +3) in SR vs non-SR were 7.7% vs 55.6%, respectively.

In a logistic regression analysis, the ORs for FOXP3, PD-1, CD68 and CD163/CD68 were 0.114, 0.176, 6.034 and 0.143, respectively ($P < 0.02$) (Table 3). Although not statistically significant, a trend of correlation with SR ($P < 0.07$) was found for high CD3 (OR, 3.643), low BTLA ILF (OR, 0.362) and high CD163 (OR, 2.669). Finally, in the multivariate model analysis, low FOXP3, low PD-1 and high CD68 correlated with SR (OR, 0.125, 0.203 and 5.983, respectively) (Table 3).

Multivariate analysis between the immune markers and cases

The samples were classified with an unsupervised hierarchical cluster analysis with the several immune markers as input variables (ie CD3, CD4, CD8, CD56, FOXP3, PD-1, BTLA, BTLA IF, TNFRSF14, CD68, CD16, CD163, PTX3 and CD11C). The result was plotted in a dendrogram and heatmap in Figure 2.B.B. The unsupervised cluster analysis could differentiate between the different entities and three main clusters were identified: cluster 1, non-SR (79.5%); cluster 2, non-SR (12.8%) and SR (70%); and cluster 3, normal mucosa (100%) and infectious colitis (80%). Therefore, the different groups could be differentiated based on the immune markers. Of note, non-SR and SR were closer,

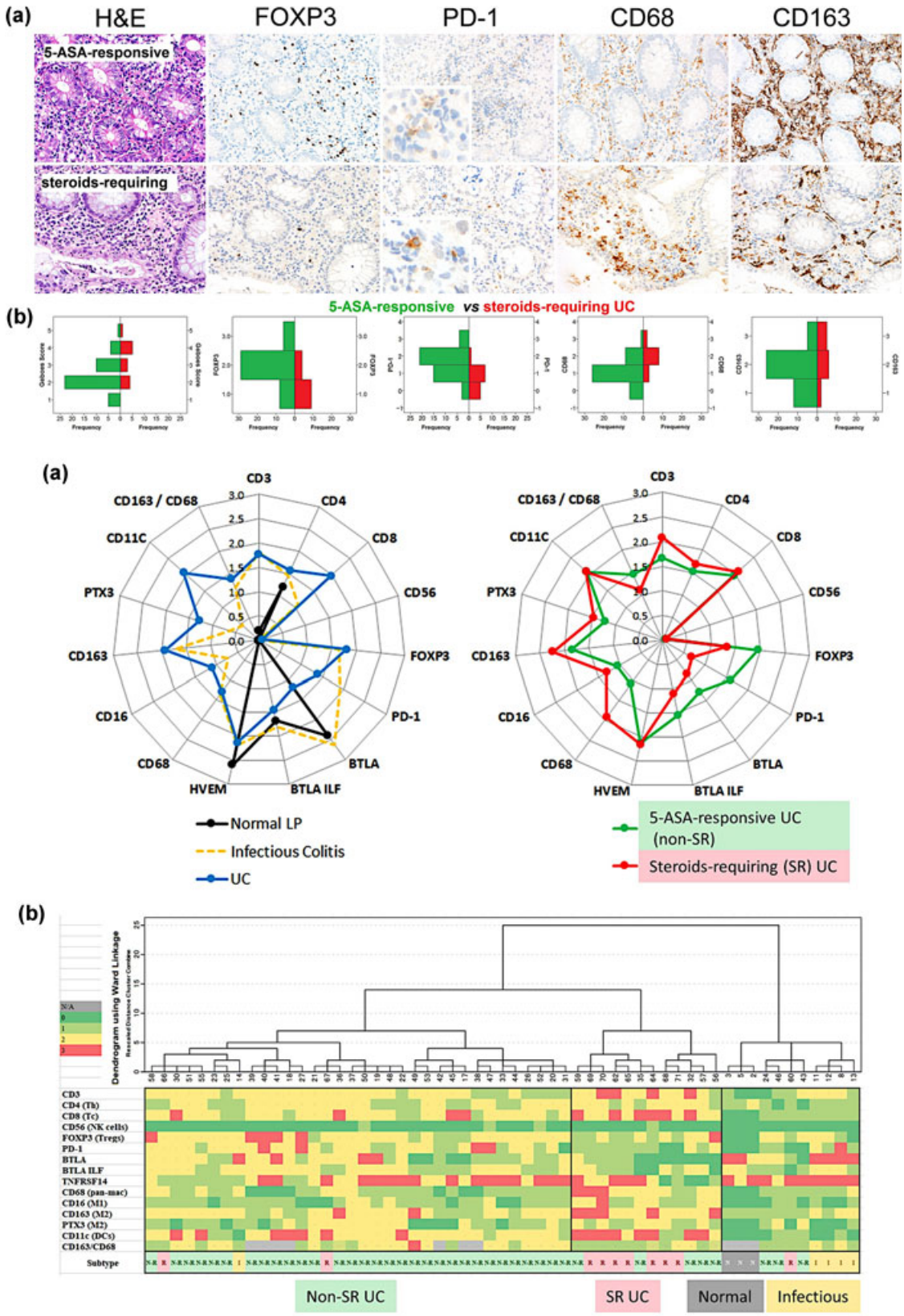


Figure 2 Continued.

Table 3 Correlation between immune markers and Ulcerative Colitis

non-SR (reference) vs steroids-requiring (SR)			
Immune marker	*P* value	Exp(B)	95% CI
Univariate analysis			
CD3	N.S. (0.054)	3.643	0.978 - 13.575
CD4	N.S.	-	-
CD8	N.S.	-	-
CD56	N.S.	-	-
FOXP3	**0.002**	**0.114**	0.029 - 0.443
PD-1	**0.002**	**0.176**	0.059 - 0.526
BTLA	N.S.	-	-
BTLA ILF	N.S. (0.066)	0.362	0.123 - 1.069
TNFRSF14	N.S.	-	-
CD68	**0.002**	**6.034**	1.906 - 19.103
CD16	N.S.	-	-
CD163	N.S. (0.064)	2.669	0.943 - 7.554
PTX3	N.S.	-	-
CD11C	N.S.	-	-
CD163/CD68 M2-ratio	**0.019**	**0.143**	0.028 - 0.729
Multivariate analysis			
FOXP3	**0.026**	**0.125**	0.020 - 0.778
PD-1	**0.044**	**0.203**	0.043 - 0.955
CD68	**0.02**	**5.983**	1.327 - 26.964

Multivariate analysis was made using the binary logistic regression, backward stepwise (conditional) method for FOXP3, PD-1, CD68 and CD163/CD68 M2-ratio. Not significant values, with the exception of "trend values", are omitted to simplify the table. SR, requiring steroid therapy.
ILF, isolated lymphoid follicle.

and separated from the cluster of normal mucosa and infectious colitis.

Gene expression analysis between active UC and noninflammatory control

We performed gene set enrichment analysis (GSEA) from an independent series of active UC and noninflammatory control because we aimed to test the same immunohistochemical markers at the gene expression level in active UC (Of note, those markers represent different immune cell populations and pathways). By immunohistochemistry we had found an increase of CD3, CD8, FOXP3, PD-1, CD68, CD16, CD163, PTX3 and CD11C but low BTLA. By GSEA we found an enrichment of genes in active UC related to Tregs, M1-like and M2-like macrophages. In addition, a functional network was created based on the markers tested by immunohistochemistry, which included secondary nodes to properly complete the pathway. When tested by GSEA the results also showed an enrichment of those markers in active UC. The five most significant genes were *CD16*, *ICAM1*, *ITGB2*, *CD86* and *CD163* (Figure 3). Therefore, at gene expression level in an independent series of active UC we can also identify an increase of immune markers and pathways.

All the Figures 1 to 3 are also present at high definition as a supplementary file (Supplementary Figures 1–5).

DISCUSSION

In this study of immunohistochemistry, we analyzed several immune markers of the mucosal immune microenvironment and immune checkpoint to understand the pathogenesis of UC and to predict the clinical evolution (ie non-SR vs SR). In addition, we correlated the immunohistochemical results with the Baron endoscopic and the Geboes histologic disease activity assessment scales, that are still valid and being used in routine diagnostic procedures.[15–17,24]

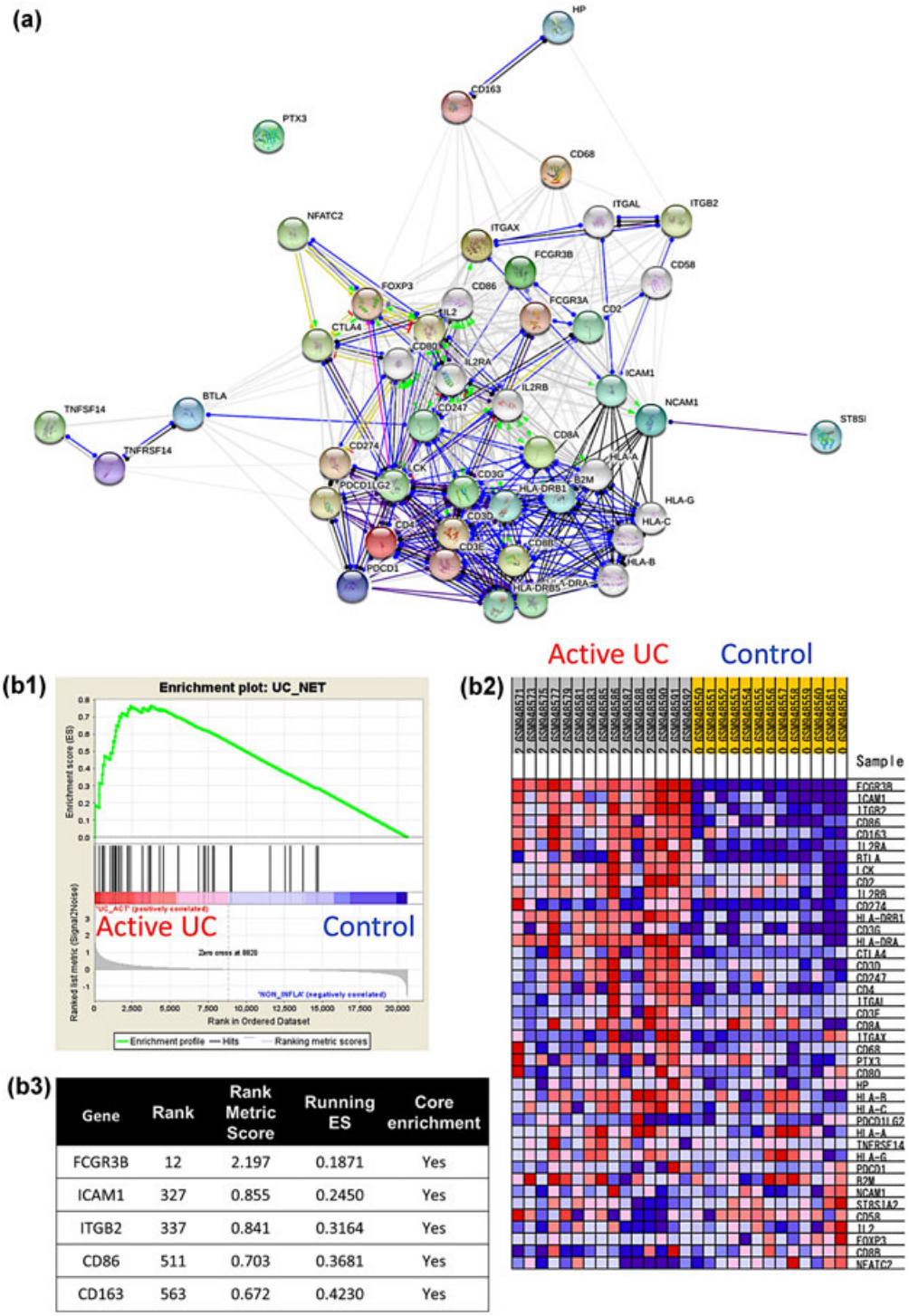
UC is a chronic idiopathic inflammatory disease of the colonic mucosa, most commonly afflicting adults aged 30 to 40 years and resulting in disability.[1–6] The pathophysiology of UC is multifactorial and in the context of the inflammatory bowel disease (IBD), which also includes Crohn's disease. The pathogenesis include factors related to the innate immunity: apoptosis of intestinal epithelial cells, increase of intestinal epithelial permeability,[25] increase of pro-inflammatory M1-like macrophages, pro-inflammatory immune response induced by DCs[26] as well as anti-inflammatory response by DCs by means of induction of Tregs,[27] increase of NK cells,[28] increase expression of defensins[29] and abnormal expression of PRRs such as TLR4.[30] The pathogenesis is also related to the adaptive immunity: Th1/Th2 balance, increased Th17 cells[31] and reduced Tregs.[32] In this project we have focused on several immune markers that characterize several components of the mucosal immune microenvironment of UC. Our results showed that, in comparison to normal mucosa, UC was characterized by an increase of T lymphocytes (CD3), Tc (CD8), Tregs (FOXP3), co-inhibitory PD-1, pan-macrophages (CD68), M1-like pro-inflammatory macrophages (CD16), M2-like anti-inflammatory macrophages (CD163 and PTX3) as well as

Figure 2 **(a).** Difference between non-steroid requiring (non-SR) and steroid requiring (SR) in ulcerative colitis (UC)**. (a)** SR UC was characterized by low expression of immune inhibitors of FOXP3 + Tregs, PD-1 receptor and CD163/CD68 M2-like macrophage polarization ratio; but high expression of CD68 + pan-macrophages. Case id.55 vs id.62. **(b)** Pyramid histogram graph of the distribution of the histologic Geboes score, FOXP3, PD-1, CD68 and CD163 between non-SR and SR in UC. **(b).** Distribution of the immune markers between groups: radar chart and multivariate analysis. **(a)** Radar chart of the comparison of the immune markers between the different entities including normal mucosa, infectious colitis and UC; and comparison between non-SR and SR subtypes. **(b)** Unsupervised clustering analysis and cluster dendrogram plot. This multivariate analysis shows that non-SR and SR UC had different expression of markers but remain closer and in a different cluster than the normal and infectious colitis. This analysis confirms our results using another statistical technique.

Table 4 Pathological mechanism and immune markers in Ulcerative colitis

	Pathological mechanism	Marker	Target cell or pathway	UC (vs normal LP)	Steroids-requiring (vs non-SR)
	Genetic predisposition	-	-	-	-
	Reduced biodiversity of active bacteria, microbial imbalance (dysbiosis)	-	-	-	-
	Increase of intestinal epithelial permeability	-	-	-	-
	Apoptosis of intestinal epithelial cells	-	-	-	-
	Random pass of luminal bacteria or food antigens through epithelial barrier	-	-	-	-
Innate immune response	Pro-inflammatory immune response induced by DCs	CD3	T lymphocytes	Increase	Trend of increase*
		CD4	T helper cells	No change	No change
	Increase of Pro-inflammatory M1-like macrophages	CD16	M1-like macrophages	Increase	No change
	Increase of anti-inflammatory M2-like $CD163^+$ macrophages (sCD163)	CD68	Pan-macrophages	Increase	Increase
		CD163	M2-like macrophages	Increase	Trend of increase*
		PTX3	M2-like macrophages	Increase	No change
		CD163/CD68	M2-like polarization	NA	Decrease
	Anti-inflammatory response by DCs by means of induction of Tregs	CD11C	Immune regulatory DCs	Increase	No change
	Increase of Natural Killer cells	CD56	Natural Killer cells	No change	No change
	Increase expression of defensins	-	-	-	-
	Abnormal expression of PRRs such as TLR	-	-	-	-
Adaptive immune response	Imbalance of $CD4^+$T lymphocytes	CD4	Th cells	No change	No change
	Increased and active cytotoxic (Tc) lymphocytes	CD8	Tc lymphocytes	Increase	No change
	Abnormal Th1/Th2 balance	-	-	-	-
	Increased Th17 cells	-	-	-	-
	Reduced Tregs	FOXP3	Tregs	Increase	Decrease
	Anti-inflammatory immune response induced by PD-1	PD-1	Co-inhibitory pathway	Increase	Decrease
	Anti-inflammatory immune response induced by BTLA	BTLA	Co-inhibitory pathway	Decrease	Trend of decrease*
	Pro-inflammatory immune response induced by TNFRSF14 stimulatory signals	TNFRSF14	Pro-inflammatory pathway	No change	No change
Stages	Phase I: pre-disease stage⇢ Phase II: acute intestinal inflammation⇢ Phase III: chronicity or resolution⇢ Phase IV: tissue destruction and complications				

-: Not evaluated; NA: non-applicable; LP: lamina propria;
*Trend is defined as P value between 0.1 and 0.05; Note: some mechanisms belong both to innate and adaptive immune responses. UC, ulcerative colitis. SR, steroids-requiring.



(b3)

Gene	Rank	Rank Metric Score	Running ES	Core enrichment
FCGR3B	12	2.197	0.1871	Yes
ICAM1	327	0.855	0.2450	Yes
ITGB2	337	0.841	0.3164	Yes
CD86	511	0.703	0.3681	Yes
CD163	563	0.672	0.4230	Yes

Figure 3 Gene Set Enrichment Analysis (GSEA) of immune markers in active ulcerous colitis, independent series GSE38713, versus normal mucosa. **(a)** Based on the markers analyzed by immunohistochemistry in this study a protein-protein network analysis was created using STRING. To properly complete the interaction network secondary nodes were added. The action types are shown in the following colors: green (activation), red (inhibition), blue (binding), yellow (transcriptional regulation) and pink (posttranslational modification). (**b1**) Subsequently, the set of genes were tested by GSEA on an independent series (GSE38713) comparing the gene expression of active UC vs. noninflammatory control. The test showed an enrichment of immune markers on active UC in a similar way as we found in our immunohistochemical study of the series of Tokai. Enrichment plot, (B2) Blue-Pink O'Gram in the Space of the Analyzed GeneSet, and (B3) GSEA details in table. The table B3 shows that *CD16 (FCGR3B)*, *ICAM1*, *ITGB2*, *CD86* and *CD163* were the five most significant markers of the leading edge, confirming the increase of macrophages in active UC.

M1-like macrophages and DCs (CD11C). This data is in concordance with the findings from the literature described above and agree with the current understanding of UC pathogenesis. TNFRSF14 and BTLA play a crucial role in preventing intestinal inflammation.[33] Normal mucosa was characterized by high TNFSRF14 and BTLA. Interestingly, UC was characterized by low expression of BTLA that is a marker of the co-inhibitory signaling pathway (ie negative regulator of T cell responses). What triggers UC is still unknown. The immune microenvironment of UC was rather like infectious colitis, therefore similar pathological mechanisms may be present. Nevertheless, UC was characterized by a more exacerbated immune response for CD8, PTX3, CD11C but lower BTLA.

UC is characterized by relapsing and remitting mucosal inflammation, starting in the rectum and extending to proximal segments of the colon. The aim of the therapy is to induce and maintain clinical and endoscopic remission. When UC flares (exacerbates) or cannot be controlled by the initial medication of 5-ASA, the patients are more likely to need other treatments such as immunosuppressants (steroids as the first choice), biological drugs or surgery.[34] Therefore, identifying prognostic factors at UC diagnosis may be helpful for the treatment and follow-up of the patients. Patients with poor prognoses tend to be young, non-smokers with elevated levels of inflammatory biomarkers [c-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)], low levels of hemoglobin, higher prevalence of extraintestinal manifestations, and extensive disease based on colonoscopy.[34] To identify immune markers related to the prognosis of the patients we stratified the patients in two groups and found that SR was characterized by lower FOXP3 + Tregs (OR, 0.114), lower PD-1 expression (OR, 0.176), higher $CD68^{+}$pan-macrophages (OR, 6.034) and lower alternatively activated M2-like macrophages as expressed by the CD163/CD68 ratio (0.143). To our knowledge, this association has not been reported so far. FOXP3 + Tregs and inhibitory receptors such as PD-1 are critical for preventing intestinal inflammation while a high component of macrophages would enhance the harmful immune response. Several in vitro and in vivo observations confirm our findings. Patients with *FOXP3* mutation (IPEX syndrome) suffer from immune-mediated colitis.[9] In a murine colitis model of severe combined immunodeficient (SCID) or recombinase activation gene knockout (RAG KO) with cell transfer of naïve $CD4^{+}CD45RB^{high}$ cells, the immune deficient mice could reverse established severe intestinal inflammation upon receiving Tregs, which interestingly accumulated in the colon. In galectin-3 knockout mice with the dextran sulfate sodium (DSS) induced colitis model, the use of galectin-3 could reverse the inflammation by inducing Tregs.[35] One of the ligands of PD-1 is the PD-L1 (CD274, programmed cell death 1 ligand 1). It has been reported that treatment with PD-L1-Fc had a protective effect on the intestinal inflammation using two murine models of inflammatory colitis, induced by DSS and T-cell transfer.[36] Monocytes and M1-like macrophages directly contribute to the defect of the barrier in IBD and large numbers of pro-inflammatory macrophages reside in the inflamed mucosa. Soluble CD163 is increased in UC patients and the treatment with anti-TNF-α normalized the sCD163 levels.[37] Macrophages, especially CD163-positive cells, express PD-L1 and PD-L2. Therefore, the immunosuppressive function of CD163 + macrophages might be mediated by PD-L1/L2. Importantly, human macrophages induce $FOXP3^{+}$Tregs via binding and rerelease of TGFB, the checkpoint inhibition.[38] Although not statistically significant, a trend of association with SR was found for high CD3 (OR, 3.643), low BTLA (OR, 0.362) and high CD163 (OR, 2.669); results that are also in agreement with the postulated pathogenesis (Table 4). Of note, distinction between non-SR and SR does not depend on one individual marker but the association of different markers, as shown in the unsupervised clustering analysis and the multivariate binary logistic regression.

Genome-wide association studies, which are technically complex and sound research projects, have identified up to 47 loci associated to UC [2]. The candidate genes provide potentially important insights into the disease pathogenesis as many of them are related to the immune system pathways such as cytokine receptor interaction, IgG binding, cytokine activity, macrophage activation and positive regulation of activated T cell proliferation. Candidate genes are *TNFRSF14 (HVEM), TNFRSF9, IL1R2, IL8RA-IL8RB, IL7R, IL10, IL12B, DAP, PRDM1 (BLIMP1), JAK2, IRF5, GNA12* and *LSP*,[12] among others. Therefore, our markers agree with the current understanding of UC susceptibility and pathogenesis. In addition, using an independent series we have also confirmed that in active colitis there is also an increase of markers that represent the same immune populations that we have studied by immunohistochemistry: in the Figure 3 we first used the markers of our project to create a functional network association analysis that allowed as to create a computational model of the immune response of the mucosa in which we can not only identify the interactions between the markers but also find new "hub" markers and know how they interact; then by GSEA we confirmed an enrichment towards active ulcerative colitis. Among the most strongly associated markers to UC we found *CD163*, a marker of M2-like macrophage polarization that we have analyzed by immunohistochemistry. In addition, we also found markers related to pro-inflammatory M1-like (*CD16*) and anti-inflammatory Tregs (*FOXP3*). We believe that this type of analysis will help us to define new pathogenic markers in the future.

In summary, we have analyzed several markers that to our knowledge have not already been described by IHC in UC. The normal intestinal mucosa was characterized by low expression of markers of the immune microenvironment but high BTLA and TNFRSF14. UC was characterized by an

increase of immune markers including CD3, CD8, FOXP3, PD-1, CD68, CD16, CD163, PTX3 and CD11C but low BTLA. The demand of steroids therapy seems to be a marker for a kind of severity of UC, SR was associated to lower expression of immune system inhibitors of FOXP3 + Tregs, PD-1 and alternatively activated anti-inflammatory M2-like macrophages (CD163/CD68 ratio); but higher pro-inflammatory CD68 pan-macrophages. In conclusion, SR seemed to be related to a lack or malfunction of immune homeostasis and mucosal immune tolerance mechanisms. Therefore, the modulation of the immune checkpoint may be relevant in the future of treatment of UC.

ACKNOWLEDGEMENTS

The authors acknowledge Yoshiko Itoh, from the Education and Research Support Center of Tokai University, School of Medicine, for the technical contribution for histological procedures.

Doctor Carreras was funded by the postdoctoral research fellowship of Tokai University, School of Medicine and by the Ministry of Education, Culture, Sports, Science and Technology-Japan (MEXT, Kaken 15K19061 and 18K15100).

DISCLOSURE STATEMENT

None Declared.

LIST OF ABBREVIATED WORDS

5-ASA	5-aminosalicylic acid, mesalamine
FFPET	formalin-fixed paraffin-embedded tissue
GSEA	gene set enrichment analysis
IBD	inflammatory bowel disease
IHC	immunohistochemistry
ILF	isolated lymphoid follicle
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
M2	macrophage M2-like polarization
non-SR	non-steroid requiring (ulcerative colitis)
OR	odds-ratio
SR	steroid requiring (ulcerative colitis)
Tc	cytotoxic T lymphocytes
Tregs	regulatory T lymphocytes
UC	ulcerative colitis

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Assessment of immune markers by immunohistochemistry.

Figure S2. Expression of immune markers in UC.

Figure S3. Difference between non-SR and SR in UC.

Figure S4. Distribution of immune markers between groups: radar chart and multivariate analysis.

Figure S5. Gene Set Enrichment Analysis (GSEA) of immune markers in active ulcerous colitis, independent series GSE38713.

Table S1A Distribution of markers of T lymphocytes and NK cells.

Table S1B Distribution of markers of immune regulation.

Table S1C Distribution of markers of macrophages and dendritic cells.

Table S2 Endoscopic Baron Score.

Table S3 Histologic Geboes Score.

Table S4 Histopathological characteristics of infectious colitis cases.