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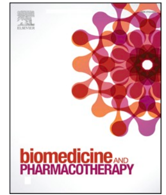

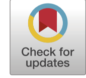

Transcriptional regulation of chemokine network by biologic monotherapy in ileum of patients with Crohn's disease

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ABSTRACT

Background: Crohn's disease (CD) exacerbation is marked by an intense cellular trafficking. We set out to determine the specific impact of biologic therapies on regulating chemokine network gene expression in healthy, mildly and severely inflamed tissue of CD patients.
Methods: Twenty CD patients on biologics (adalimumab, ustekinumab, vedolizumab) or untreated undergoing colonoscopy due to clinical symptoms of flare. Healthy, mildly and severely inflamed ileum biopsies from each patient were collected. Chemokines and receptors gene expression was analyzed and a STRING analysis for functional enrichment was performed.
Results: The chemokine network exhibited wide transcriptional differences among tissues in active untreated patients, whereas all biologic treatments reduced these differences and homogenized their transcriptional activity. In mildly inflamed tissue, all treatments showed gene upregulation while ustekinumab additionally maintained the downregulation of genes such as CCL2, CCL3, CCL17 or CCL23, involved in T cell chemotaxis, inflammatory monocyte and NK trafficking. In severely inflamed tissue, all treatments shared a downregulatory effect on chemokines controlling T cell response (i.e. CXCL16, CXCR3). Adalimumab and vedolizumab significantly reduced the expression of genes promoting antigen presentation by DCs and the initiation of leukocyte extravasation (i.e. CXCL12, CCL25, CCR7). Ustekinumab significantly reduced genes positively regulating Th1 cytokine production and IL-8 mediated signaling (i.e. IL1B, XCL1, CXCR1, CXCR2).
Conclusion: Biologic therapies differentially target the chemokine network gene expression profile in the ileal tissue of active CD patients. These results may contribute to better understanding cell homing and to defining future personalized therapeutic strategies for CD patients.

1. Background

Crohn's disease (CD) is a major form of inflammatory bowel disease with a relevant incidence worldwide [1]. The genetic background, several environmental factors and changes in the microbiota content contribute to an immune dysregulation that allows the onset and the progression of this chronic autoinflammatory disease. CD is characterized by transmural inflammation occurring anywhere along the alimentary tract and, despite the alternating periods of remission with spontaneous inflammatory flares, CD is considered a progressive and

Abbreviations: IBD, Inflammatory bowel disease; CD, Crohn's disease; IL, interleukin; GOI, genes of interests.

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destructive condition, deeply affecting patients' quality of life [2].

Inflammation constitutes a multifaceted process in which leukocyte recruitment is required to deploy an efficient, coordinated immune response [3]. In turn, this cellular traffic also requires a tight control to prevent excessive recruitment and, therefore, a sustained inflammatory environment, a key driver of disease complications [4]. In command of cellular recruitment and recirculation during inflammatory processes, chemokines and their receptors stand out as a versatile network of mediators timely orchestrating individual's evolving local and systemic immune status. Chemokines are a large family of cytokines with biological functions beyond cell migration regulation, as summarized in the past [5]. They can operate by inducing soluble gradients to generate chemotactic mobilization or inducing integrin activation in leukocytes [6,7].

The use of anti-TNF-α has significantly improved the therapeutic outcomes in CD. Biologic therapies have expanded in the last years to other molecules such as anti-IL12/IL23 or anti-α4β7 integrin among others, interfering with different and specific aspects of the immune responses [4]. Despite this array of possibilities, improving efficacy of treatments still constitutes a goal required to reduce the number of patients non-responding or showing loss of response to those therapies. Roughly, one third of patients treated with anti-TNF-α don't show any response to this therapy [8]. Furthermore, approximately 30–50% of CD patients lose response to adalimumab during disease progression, and a rate of 13% of loss of response (requirement of intensified schedules) has been estimated per patient-year follow-up, according to a meta-analysis performed by Gisbert et al. [9]. In the recent open label long-term extension of the randomized double-blind placebo-controlled IM-UNITY study [10], approximately 44% of anti-TNF naïve patients treated with ustekinumab maintained clinical remission at five years in the intend-to-treat analysis [11]. In the case of vedolizumab, 43% of patients remained in remission at three years in the intend-to-treat analysis, as described in the GEMINI long term safety trial [12].

Considering these facts, and that biologic therapies specifically target the immune system through different strategies, a more comprehensive knowledge on treatments effects over particular aspects at a molecular level could help first, understand bypassed immune pathways associated with lack of treatment efficacy and second, design personalized strategies to better manage CD patients in the future. In this study, we set out to determine the specific impact of biologic monotherapies on regulating chemokine network gene expression in healthy, mildly and severely inflamed ileum of CD patients.

2. Patients and methods

2.1. Patients and study design

Consecutive patients diagnosed of CD, established according to standard clinical, endoscopic, histological and radiographical criteria [13] and managed at the IBD Unit of Hospital General Universitario de Alicante, undergoing colonoscopy due to clinical symptoms of flare were considered for inclusion in this prospective observational study. Inclusion criteria were aged between 25 and 50 years-old, a regular regime of adalimumab, ustekinumab or vedolizumab as monotherapy for at least 6 months, the absence of flares in the previous 6 months, and a signed informed consent to participate. Exclusion criteria were the use of combined therapies, the stable use of biologic monotherapy for less than six months, disease flares in the previous six months, age younger than 25 and older than 50 years-old, and refusal to sign informed consent. A subgroup of age-matched, debuting CD patients was included as untreated control group.

Ileum biopsies were collected from 20 CD patients ($n = 5$/treatment group) and preserved in formol and RNAlater. Biopsies from healthy (no ulcers), mildly (scattered ulcers < 5 mm) and severely (ulcers > 5 mm) inflamed ileum segments were obtained from all patients and confirmed in the written histopathology reports. In healthy samples, non-inflamed tissue with normal architecture. In mildly inflamed samples, architectural alteration and acutely inflamed tissue, with infiltrating intraepithelial leukocytes. In severely inflamed samples, ulcerated and granulated tissue. Clinical and analytical characteristics of patients were recorded at inclusion in the study. Disease clinical activity was determined by Crohn's disease activity index (CDAI) > 150 and presence of clinical symptoms of relapse. Disease endoscopic activity was determined by Simple Endoscopic Score for Crohn Disease (SES-CD). All patients were Caucasian of Mediterranean ethnicity and were classified according to the Montreal classification. All included patients received diaries to record symptoms 1 week before inclusion and sample collection, and signed an informed consent to participate in the study. The study was approved by the Ethics Committee of Hospital General Universitario and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All patients gave their informed consent prior to their inclusion in the study.

2.2. Serum drug levels

Enzyme-linked immunosorbent assays (ELISAs) were carried out to measure free adalimumab (Matriks Biotek, Ankara, Turkey), ustekinumab and vedolizumab (IDK AG, Bensheim, Germany) levels according to the manufacturers' instructions. Detection limits for each biologic therapy were 0.3 ug/mL (adalimumab), 0.9 ng/mL (ustekinumab) and 7.4 ng/mL (vedolizumab).

2.3. mRNA isolation and RT-PCR

RNA was isolated using RNeasy kit (Qiagen, Heidelberg, Germany) according to manufacturer's instructions. Gene expression of human chemokines and their receptors was analyzed by using *RT² Profiler™ PCR Array Human Chemokines & Receptors* (GeneGlobe ID – PAHS-022Z, Qiagen) on a CFX Connect Real-Time PCR System (Biorad, Hercules, CA). Raw data related to this study has been deposited in Mendeley Data and is citable and publicly available: Lopez-Atalaya, Jose P.; Frances, Ruben (2021), "BioMnoTrpyCD", Mendeley Data, V1, doi: https://doi.org/10.17632/s4r6xfdhjs.1.

2.4. Gene expression analysis

Gene expression was quantified by real-time PCR amplification cycles, and fold-change calculations or gene expression ratios were calculated using the classic, well-established, and widely adopted $\Delta\Delta C_T$ method. Genes without detection or with detection higher than 35 amplification cycles were replaced to a value of 35. A total of 84 genes of interest (GOI) were normalized to the average of 5 Housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, ACTB), using the formula: $\Delta C_T = C_T$ (GOI) − average (C_T (Housekeeping genes)). The average ΔC_T was calculated using the formula: Average ΔC_T = (ΔC_T (Sample1) + ΔC_T (Sample2) + ... ΔC_T (Sample n))/nSamples. For each tissue state, the $\Delta\Delta C_T$ value was calculated by subtracting the ΔC_T value of each Test group from the Control group: $\Delta\Delta C_T = \Delta C_T$ (Test Group n) − average ΔC_T (No treatment, healthy tissue). The fold-change (FC) value was then calculated by converting the $\Delta\Delta C_T$ from a $\log_2$ scale to a linear scale using the following equation: $FC = 2^{(-\Delta\Delta C_T)}$. Downregulated FC values were further transformed to negative linear scale: $-1/FC$. To determine gene patterns, a matrix with normalized gene expression (ΔC_T) for all the samples was built. Gene expression was centered and scaled (z-score), and a distance matrix between them was computed using the Euclidean distance measure. Hierarchical clustering was performed between the genes, with a cut in 12 clusters. Finally, 5 groups were manually merged by similar patterns resulting into 7 curated clusters. CCL14 gene was downstream removed because of its low level and abnormal pattern of expression (high distance to the rest of genes).

2.5. Hierarchical clustering heatmap

To study the relationship between treatment, tissue and patients, we generated a clustered heatmap. Fold-changes were obtained using healthy tissue from non-treated patients. Housekeeping genes were removed and remaining fold-change values were centered and scaled (z-score). Pearson correlation over z-score of FC values was used as a distance measure to perform a hierarchical clustering for rows and columns by average (UPGMA) agglomeration method.

2.6. Statistical analysis

Friedman tests were conducted on normalized RT-qPCR data (ΔCT) to study differences in gene expression for tissue states in each group of treatment, followed by post hoc paired Wilcoxon signed-rank test with Bonferroni correction for multiple testing (Supplementary Table 1). Genes with p value < 0.05 (Friedman test) and p.adj value < 0.05 (paired Wilcoxon signed-rank test with Bonferroni correction) were considered significantly differentially expressed.

Kruskal-Wallis tests were conducted on normalized RT-qPCR data (ΔCT) to study differences in gene expression for treatment effect in each type of tissue state, followed by post hoc Wilcoxon rank sum test with Bonferroni correction for multiple testing (Supplementary Table 2). Genes with p value < 0.05 (Kruskal-Wallis test) and p.adj value < 0.05 (Wilcoxon rank sum test with Bonferroni correction) were considered significantly differentially expressed.

2.7. Gene set enrichment analysis on immune cells and their biological process

We used the STRING (version 11.0) tool on identified genes to determine protein-protein interactions and to perform an enrichment analysis according to the Gene Ontology (GO) biological process classification in "*Homo sapiens*" species. We considered a strength value ($\log_{10}$[observed/expected]) above 2 as well as implicated immune cell type when evaluating the enrichment effect.

3. Results

3.1. Clinical and analytical characteristics of patients

Table 1 shows clinical and analytical characteristics of included patients, distributed by different biologic monotherapies. Clinical and endoscopic activity was confirmed by CDAI and SES-CD, respectively. None of patients were receiving steroids. None of them had perianal activity, showed extraintestinal manifestations or had undergone previous surgery. Median [min-max] serum drug levels for different biological therapies were 6.6 [4.1–7.4] μg/mL (adalimumab), 0.8 [0.6–2.1] μg/mL (ustekinumab), and 13.6 [11.6–18.9] μg/mL (vedolizumab), respectively.

3.2. Chemokine and chemokine receptor profiling in CD

Gene expression of chemokine and chemokine receptors were evaluated in healthy, mildly and severely inflamed ileum segments of CD patients under different biologic monotherapies. Fig. 1A shows an example of healthy, mildly and severely inflamed segments evaluated in each patient. Progressive architectural alteration and increasing leukocyte intraepithelial infiltration are evidenced histologically (Fig. 1B).

Genomic data showed a close clustering of treatments and tissues along the series of CD patients, suggesting that differences in gene expression are due to treatment and tissue effect rather than inter-individual variability (Fig. 2A). The Principal Component Analysis (PCA) showed that both tissue and treatments individually display an effect on chemokine and chemokine-related gene expression profile. In addition, the bidimensional chart showed a distinct behavior for severely inflamed tissue in patients without treatment (Fig. 2B).

Changes between tissues in the expression of chemokine-related genes were frequent among patients without treatment (87%). Biologic monotherapies were able to homogenize gene expression levels

Table 1
Clinical and analytical characteristics of patients.

	Untreated patients (n = 5)	Adalimumab-treated patients (n = 5)	Ustekinumab-treated patients (n = 5)	Vedolizumab-treated patients (n = 5)
Gender, male (%)	3 (60%)	2 (40%)	3 (60%)	3 (60%)
Age (years)	35 ± 10	42 ± 9	45 ± 8	40 ± 10
Smoking habit, yes (%)	2 (40%)	1 (20%)	2 (40%)	1 (20%)
CDAI	240.2 ± 68.5	185.5 ± 32.8	210.3 ± 59.4	192.8 ± 38.6
SES-CD	11 ± 3	9 ± 2	10 ± 2	10 ± 2
Disease duration (months)	1 ± 2*	65 ± 45	72 ± 56	68 ± 42
Previous surgery, yes (%)	–	–	–	–
Montreal classification, n (%):				
Age of onset				
A1	–	–	–	–
A2	5 (100%)	5 (100%)	5 (100%)	5 (100%)
A3	–	–	–	–
Location				
Ileal	3 (60%)	2 (40%)	4 (80%)	3 (60%)
Ileocolonic	2 (40%)	3 (60%)	1 (20%)	2 (40%)
Colonic	–	–	–	–
Behavior				
1	4 (80%)	3 (60%)	3 (60%)	4 (80%)
2	1 (20%)	2 (40%)	2 (40%)	1 (20%)
3	–	–	–	–
Perianal activity, yes (%)	–	–	–	–
EIM, yes (%)	–	–	–	–
Use of steroids, yes (%)	–	–	–	–
Total leukocytes (mm3)	6946 ± 2140	6384 ± 1996	7142 ± 2251	6686 ± 2366
Hemoglobin (g/dL)	13.8 ± 1.4	13.9 ± 2.1	14.0 ± 2.3	13.7 ± 1.5
Albumin (g/dL)	3.9 ± 0.6	4.0 ± 0.5	4.1 ± 0.8	4.2 ± 0.6
CRP (mg/dL)	1.3 ± 0.6	0.9 ± 0.3	1.1 ± 0.4	1.0 ± 0.4
Fecal calprotectin (ug/g)	648 ± 256.2	664.9 ± 138.4	610.1 ± 141.2	676.5 ± 132.6

CDAI: Crohn's disease activity index; SES-CD: Simple endoscopic score for Crohn's disease; EIM: extra intestinal manifestations; CRP: C-reactive protein.
* $p < 0.01$ compared to the rest of groups.

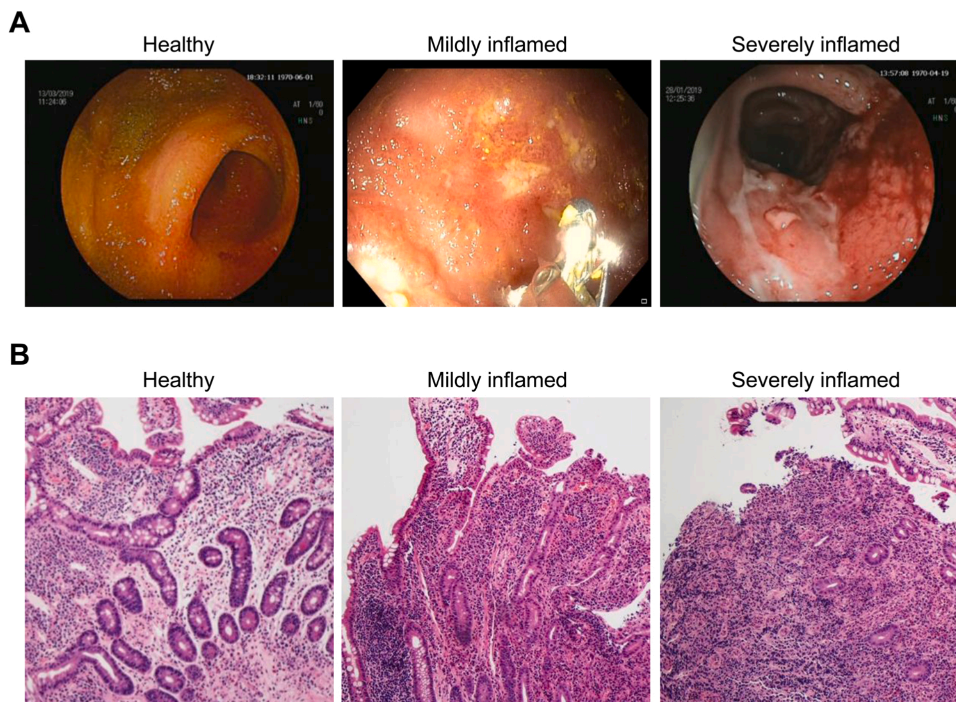

Fig. 1. (A) Examples of healthy, mildly inflamed and severely inflamed ileal sections from a representative CD patient. Samples from the three sections were obtained from each included patient. (B) Examples of histologies from collected ileal sections from a representative CD patient. In healthy samples, non-inflamed tissue with normal architecture. In mildly inflamed samples, architectural alteration and acutely inflamed tissue, with infiltrating intraepithelial leukocytes. In severely inflamed samples, ulcerated and granulated tissue.

among tissues, reducing the number of genes changing their expression levels compared to non-treated tissues (Fig. 3A). Chemokine-related genes in each tissue were grouped into 7 clusters according to their common expression patterns for each treatment (Fig. 3B). Of relevance, treatments are associated with an effect on healthy tissue, modifying the gene expression levels in certain clusters. While several treatments on mild inflamed tissue increased gene expression in clusters such as 3, 6 or 7, treatments on severely inflamed tissue reduced gene expression levels in most clusters.

3.3. *Treatments effect over chemokine network in healthy, mildly and severely inflamed tissue*

We then evaluated the genes significantly changed, as well as the functional enrichment of these genes in immune regulation pathways and cellular chemotaxis, for each treatment versus non-treatment in healthy, mildly and severely inflamed states.

As observed, adalimumab and ustekinumab regulatory intervention over chemokine network in healthy tissue comprises more genes than that induced by vedolizumab when compared with untreated tissue (Fig. 4A). Adalimumab mainly allows an increase in chemokine-related genes expression, except for CCL14 and CXCL2, which mediates neutrophil trans-endothelial migration in response to TNF-alpha. However, ustekinumab shows a set of genes regulated differently. While chemokines such as CCL25 and its receptor CCR9 show an increased expression, others such as CCL1 and its receptor CCR8 are downregulated. Vedolizumab shows the lowest impact on healthy tissue, upregulating a few genes as for instance CCL25 and its receptor CCR9.

All three treatments were able to similarly regulate chemokine mediated signaling pathways, affecting lymphocyte migration in healthy tissue (Fig. 4B). Although adalimumab and ustekinumab also regulated myeloid leucocyte migration, ustekinumab downregulated CCL26, CCL17, CCL3 and XCL1 whereas adalimumab downregulated CCL17 and CCL24. Vedolizumab induced an upregulation in genes involved in cellular defense response such as CXCL9. In addition, ustekinumab also reduced leukocyte extravasation and dendritic cell (DC) apoptosis, as shown by the upregulation of CCL21 and CXCL12, which are involved in the negative regulation of both leukocyte tethering and rolling and DC apoptosis.

Functional enrichment in cellular chemotaxis revealed significant changes in genes related to trafficking of main leukocyte populations for all three treatments compared to untreated healthy tissue (Fig. 4C). While Adalimumab and vedolizumab induced a significant upregulation in genes related to trafficking of main leukocyte populations, ustekinumab induced a mixed regulatory pattern among genes changed compared to untreated healthy tissue. Under this treatment, a set of chemokine-related genes involved in lymphocyte, monocyte and neutrophil chemotaxis (XCL1, CCL3, CCL16, CCL17 and CCL26) was downregulated compared to untreated healthy tissue.

The modification of chemokine-related gene expression pattern in mildly inflamed tissue was similar to that induced over the healthy tissue for each treatment (Fig. 5A). While all modifications associated with adalimumab and vedolizumab treatments were upregulatory, ustekinumab showed a mixed down/upregulatory pattern, where genes such as CCL2, CCL3 or CCL17 maintained their downregulation, as already observed in healthy tissue. Besides, others such as CCL23, involved in chemotaxis of resting T cells and monocytes rather than neutrophils, were also downregulated.

Treatments effect in mildly inflamed tissue was like that observed in healthy tissue, though a wider array of pathways involved in inflammatory response to antigens was upregulated in the functional enrichment analysis in the case of adalimumab compared to non-treated patients. Genes implicated in the regulation of T cell migration are similarly modified by all treatments. Interestingly, all three biologics upregulated the expression of genes involved in NOS biosynthetic process (Fig. 5B).

Compared with non-treated patients, majority of changes induced by vedolizumab belonged to a subset of genes affecting T cell chemotaxis. Adalimumab and ustekinumab also showed a significant modification of genes regulating monocyte populations, and to a lesser extent NK and eosinophils. On the contrary, genes implicated in the regulation of neutrophil migration are similarly modified by all treatments (Fig. 5C).

As shown in Fig. 6, and opposite to changes generally induced in healthy and mildly inflamed tissue, all gene modifications induced by treatments in severely inflamed tissue were downregulatory. Molecules such as CCL25, an inducer of pro-migratory responses and the activation

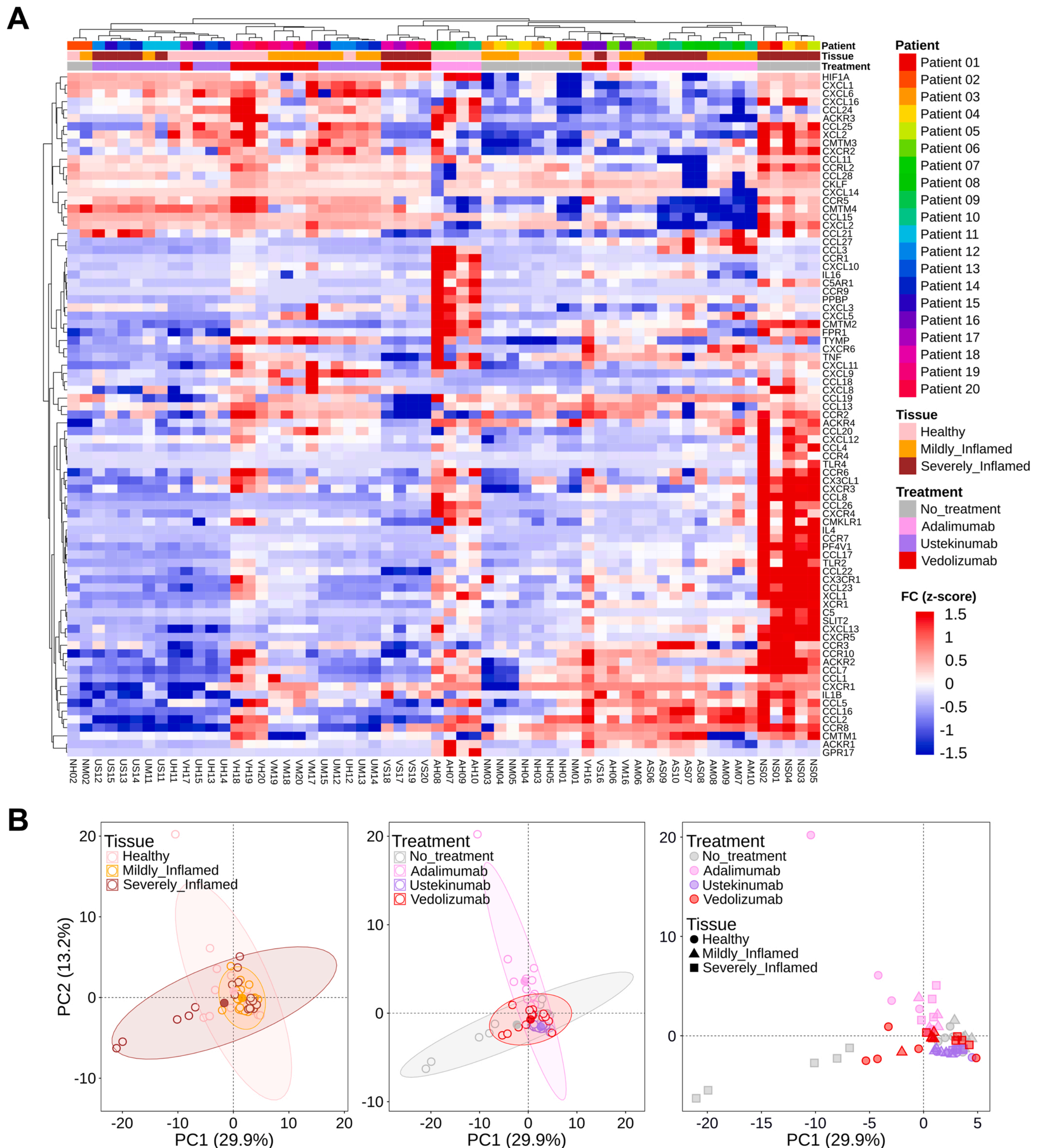

Fig. 2. (A) Heatmap with z-score of fold change showing the expression profile of chemokine and chemokine receptor genes in all samples from all included patients (n = 20). (B) Principal Component Analysis (PCA) showing the distribution of patients' samples either by tissue (left) or treatment (center), and bidimensional chart of the gene expression dataset for each sample (right).

of α4β7- and α4β1-integrins, or CCL1, expressed in activated T cells and chemotactic for monocytes, among others, were significantly downregulated by all three treatments.

In addition to the downregulation of genes participating in lymphocyte migration by all three treatments, adalimumab and vedolizumab significantly reduced the expression of genes promoting antigen presentation by DCs and the initiation of leukocyte extravasation. On the other hand, ustekinumab significantly reduced genes positively regulating Th1 cytokine production and IL-8 mediated signaling. Ustekinumab and vedolizumab also decreased the expression of genes activating the cellular response to MCSF. Regarding genes involved in cellular chemotaxis, all treatments show a similar ability in reducing the expression of several genes related to different cell type chemotaxis, including lymphocytes, monocytes and neutrophils. This is probably due

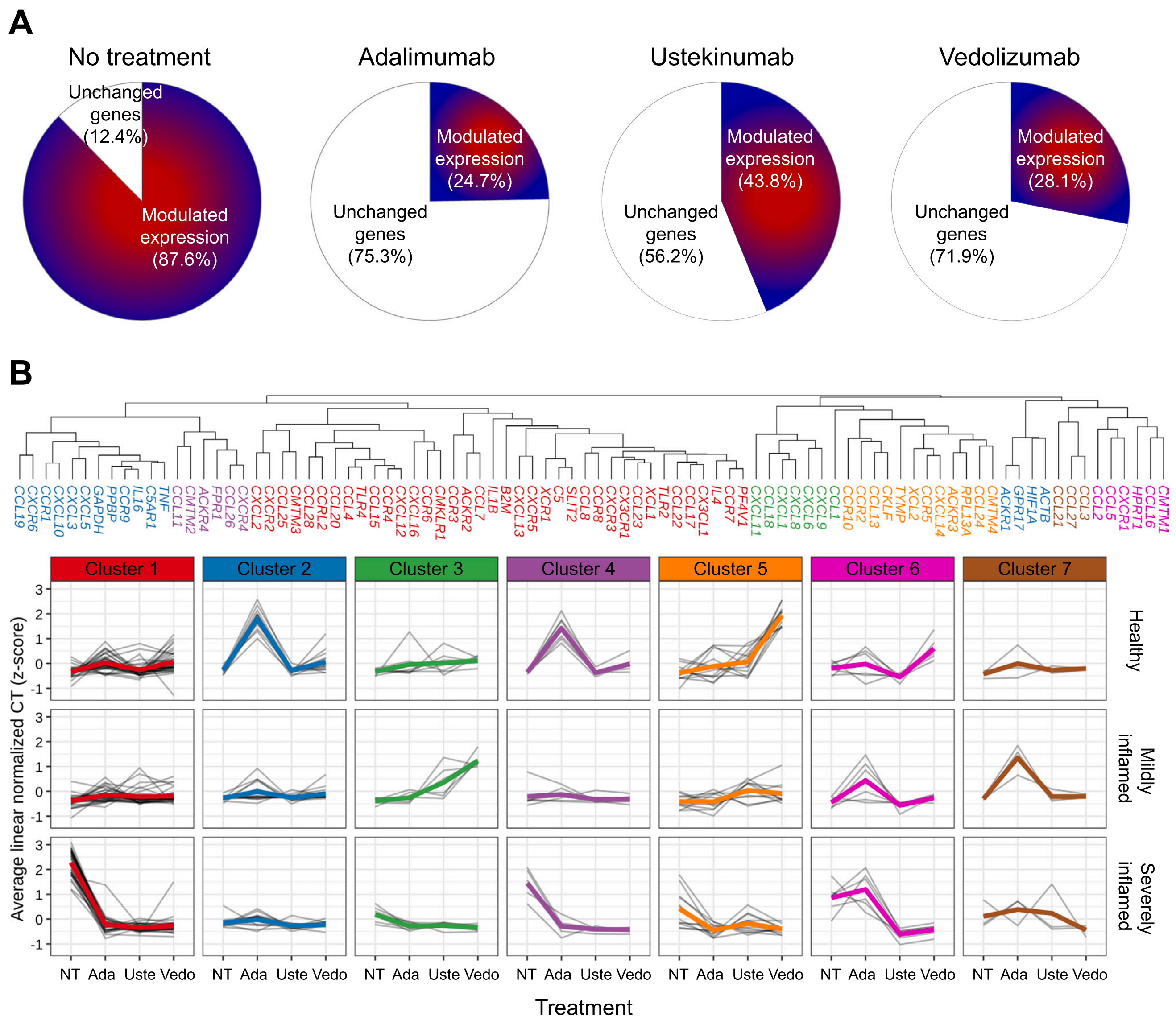

Fig. 3. (A) Significant changes among tissue states in the expression of chemokine-related genes for non-treated patients and for patients with each individual treatment (n = 5 per treatment group). (B) Hierarchical clustering analysis of chemokine and chemokine receptor genes for the different tissues according to their common expression patterns for each treatment. NT: no treatment; Ada: adalimumab; Uste: ustekinumab; Vedo: vedolizumab.

to an abundant set of chemotactic-related genes regulated by all three treatments. The functional enrichment analysis was able to identify significant reductions in the expression of genes involved in macrophage and eosinophil chemotaxis for adalimumab and ustekinumab treatments compared to untreated, severely inflamed tissue.

Finally, Table 2 shows a signature of chemokine and chemokine receptor genes exclusively modulated by each biologic therapy compared with non-treated patients to provide a list of unique, treatment-specific targets in each tissue. Genes that remained unaltered by any biologic treatment in any tissue are also listed.

4. Discussion

The present study provides a comparative evaluation of the biologic monotherapy effect on the chemokine gene network expressed in healthy, mildly and severely inflamed ileum of flared CD patients. While all treatments reduced the transcriptional differences among tissues and modulated the gene expression of chemokines and their receptors compared with untreated patients, regulatory patterns, functional pathways and cell types targeted show specific differences among treatments. These results may contribute to defining future personalized therapeutic strategies in CD patients.

Biologic therapeutic options for CD patients have expanded in the last years and have contributed to a better clinical management of patients [14]. Along with this increment, the need for a deeper knowledge on their interactions with patients' immune system has been raised, as response to these treatments continues to be improvable and selecting a suitable first-line strategy may have important benefits for patients [15]. In this regard, chemokines have been proven to regulate the leukocyte response during inflammation through their inflammatory and/or homeostatic functions [16].

Different gut locations show specific chemokine profiles, finely orchestrating cell recruitment to these sites and contributing to define different regional immune responses [17]. It is important to stress that all three therapeutic molecules homogenized the transcriptional levels of chemokine and chemokine-receptor genes among tissues, reducing the large number of changes observed between healthy, mildly and severely inflamed tissue in untreated patients. This fact is probably due to the wide downregulation induced in severely inflamed tissue by all biologic molecules. Although this supports at least a partial patients'

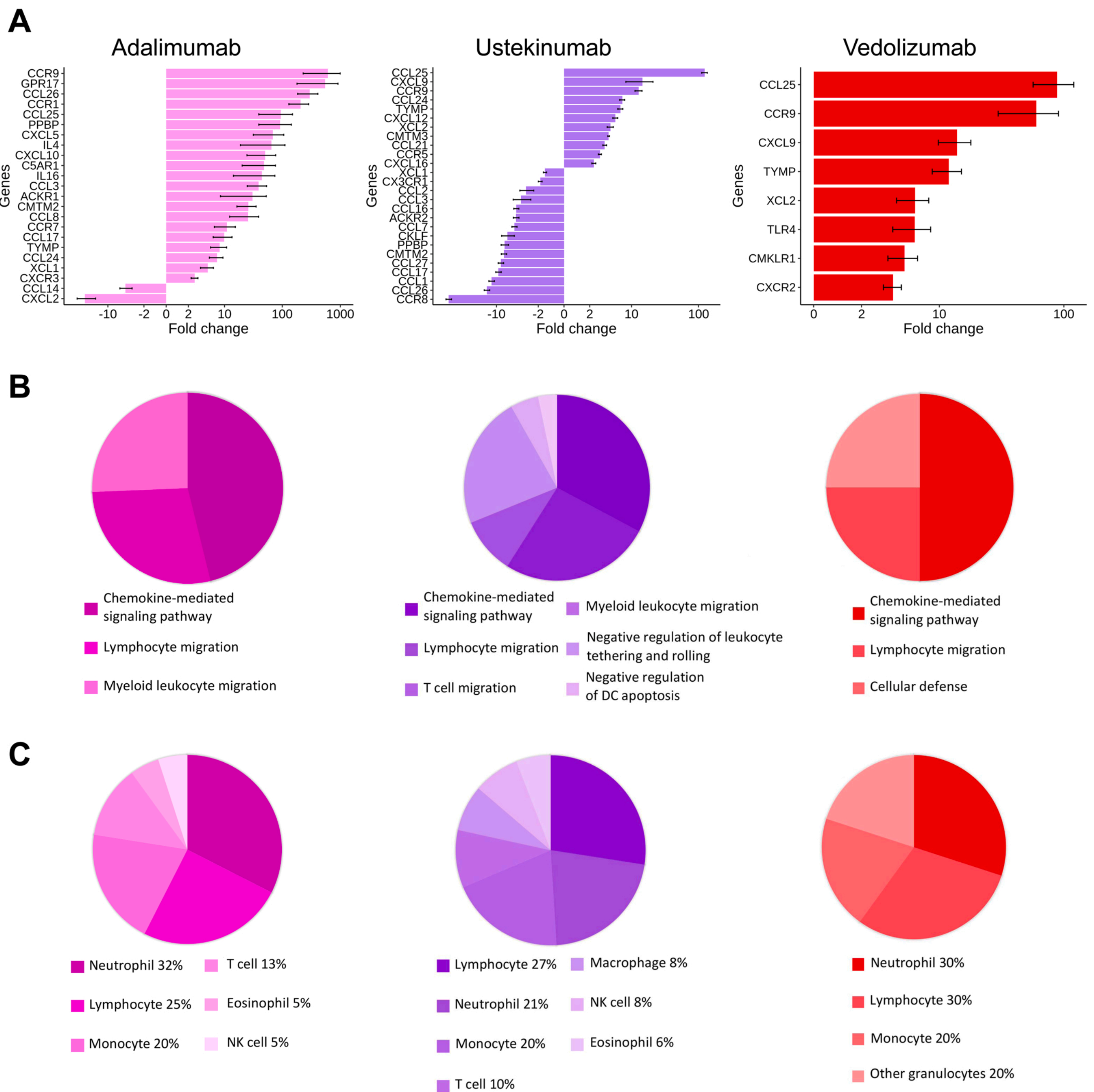

Fig. 4. (A) Genes significantly changed by each treatment vs no treatment in healthy ileal tissue. Bar plots show mean fold change ± standard errors of the mean (s.e.m.) (n = 5 per treatment group). (B) Functional enrichment of genes involved in immune regulation by each treatment vs no treatment in healthy tissue. (C) Functional enrichment of genes involved in cellular chemotaxis by each treatment vs no treatment in healthy tissue.

immune response to their individual treatments, the fact that these patients have a flare may be reflecting that this modulation is not preserving tissue homeostasis from either sporadic or recurrent triggers at specific intestinal locations [1]. Therefore, the inflammatory microenvironment in these sites, in which tissue-resident memory T cells may be present [18], turn out as a relevant aspect in terms of treatment efficacy and would support the segmented manifestation of disease even under systemic therapies. It is also interesting to observe how chemokine-related genes are grouped into different clusters according to their transcriptional behavior in each tissue. Getting to know isolated and combined functions of these genes in immune cell trafficking may be of interest for predicting treatment effects on these functions, as it happens for instance with the induction of IL-7R signaling pathway in T cells observed in IBD patients who have failed to respond to therapy with anti-TNF-α or anti-α4β7 [19].

The comparison of individual treatments with untreated samples for each specific tissue also provides valuable data on modulation requirements. Patients on adalimumab or vedolizumab show a chemokine upregulatory pattern in healthy and mildly inflamed tissue upon flare. This profile would correspond to an initial inflammatory status in which treatments are not yet able to reduce T cell gut homing, as shown by the upregulation of CCL25 and its receptor CCR9 and CCL20 and its receptor CCCR6, or neutrophil recruitment as shown by CXCL1 an CXCL5 in healthy and mildly inflamed tissues. These molecules have been implicated in inflammatory activation in IBD. An increased number of circulating $CCR9^{+}$ T cells is present in patients with small bowel

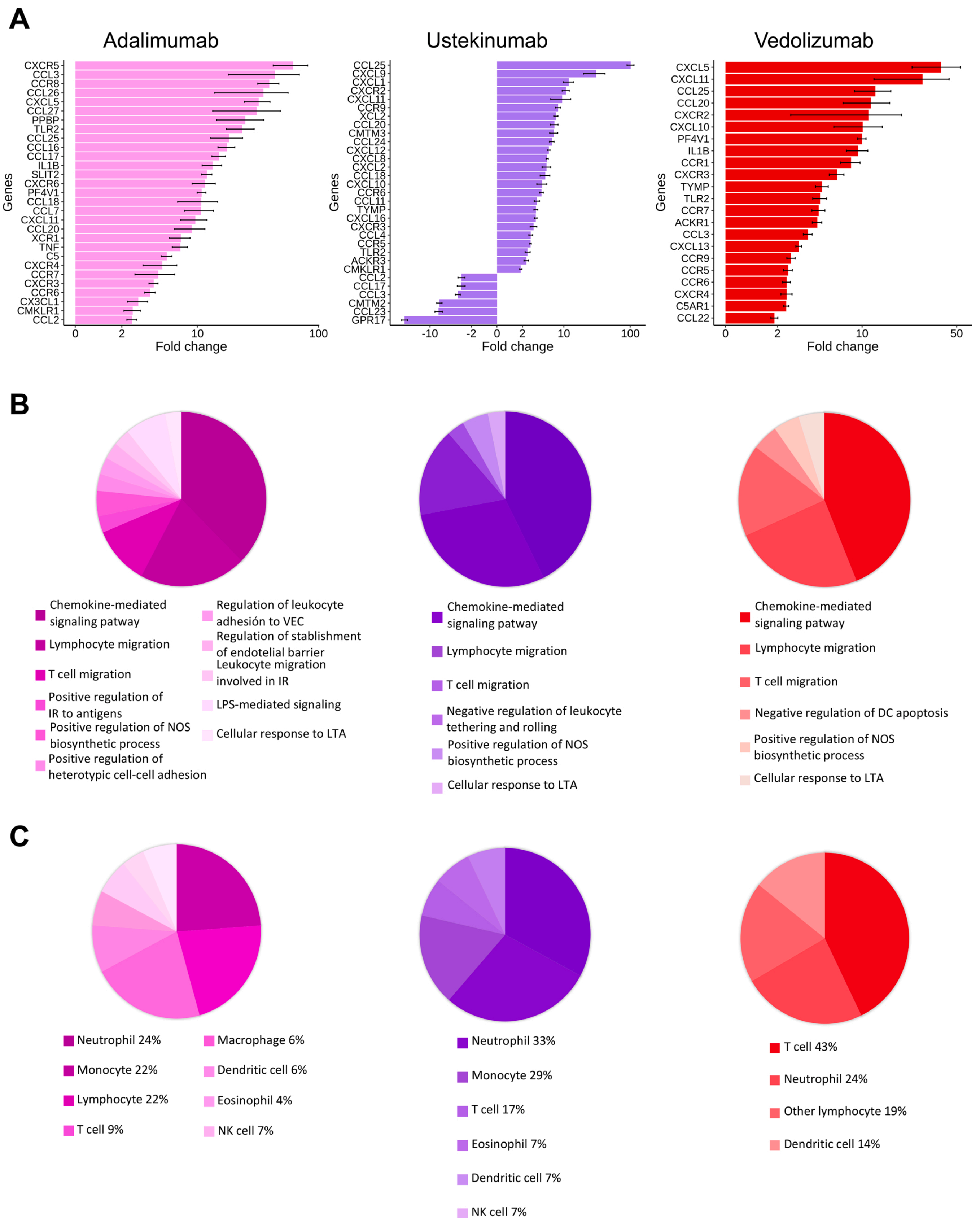

Fig. 5. (A) Genes significantly changed by each treatment vs no treatment in mildly inflamed tissue. Bar plots show mean fold change ± standard errors of the mean (s.e.m.) (n = 5 per treatment group). (B) Functional enrichment of genes involved in immune regulation by each treatment vs no treatment in mildly inflamed tissue. (C) Functional enrichment of genes involved in cellular chemotaxis by each treatment vs no treatment in mildly inflamed tissue. IR: inflammatory response; NOS: nitric oxide synthase; VEC: vascular endotelial cell; LPS: lipopolysaccharide; LTA: lipoteichoic acid.

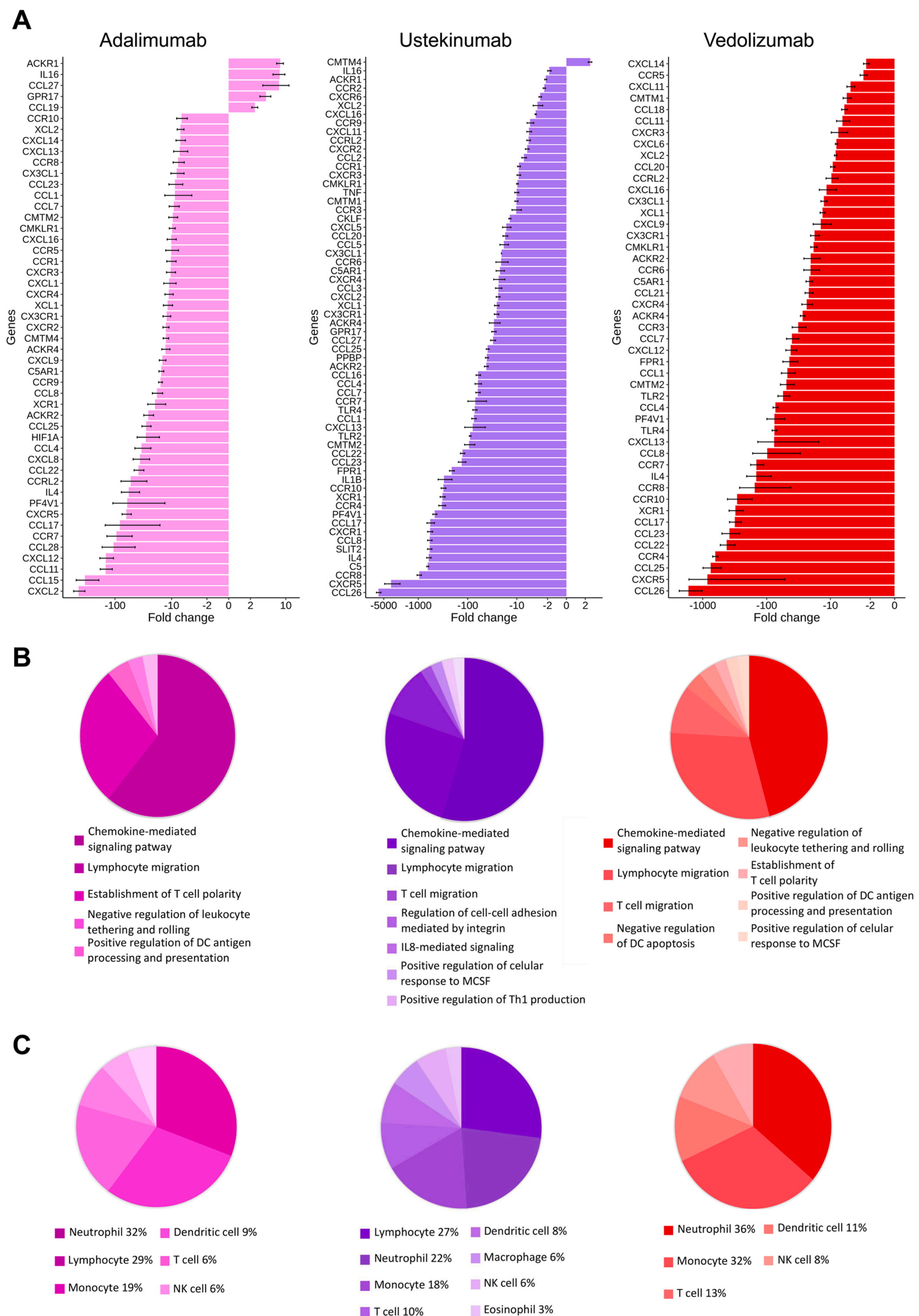

Fig. 6. (A) Genes significantly changed by each treatment vs no treatment in severely inflamed tissue. Bar plots show mean fold change ± standard errors of the mean (s.e.m.) (n = 5 per treatment group). (B) Functional enrichment of genes involved in immune regulation by each treatment vs no treatment in severely inflamed tissue. (C) Functional enrichment of genes involved in cellular chemotaxis by each treatment vs no treatment in severely inflamed tissue. MCSF: macrophage colony stimulating factor.

Table 2
Signature of treatment-exclusive chemokine and chemokine receptor gene targets in each tissue. Red colored: significantly upregulated genes compared to untreated patients; blue-colored: significantly downregulated genes compared to untreated patients. Ada: adalimumab; Uste: ustekinumab; Vedo: vedolizumab.

Genes modulated by biologic therapy *vs* no treatment									Genes unaltered by any biologic therapy in any tissue		
Healthy			Mildly inflamed			Severely inflamed					
Ada	Uste	Vedo	Ada	Uste	Vedo	Ada	Uste	Vedo	Ada	Uste	Vedo
ACKR1	ACKR2	CMKLR1	C5	ACKR3	ACKR1	CCL15	C5	CCL18	ACKR3	CCL13	ACKR3
C5AR1	CCL1	CXCR2	CCL16	CCL11	C5AR1	CCL19	CCL16	CCL21	CCL13	CCL14	CCL13
CCL14	CCL16	TLR4	CCL26	CCL23	CCL22	CCL28	CCL2	CXCL6	CCL21	CCL15	CCL14
CCL8	CCL2		CCL27	CCL24	CCR1	CXCL1	CCL3		CCL5	CCL19	CCL15
CCR1	CCL21		CCL7	CCL4	CXCL13	CXCL8	CCL5		CCR2	CCL28	CCL16
CCR7	CCL27		CCR8	CMTM2		HIF1A	CCR2		CCR3	CXCL14	CCL19
CXCL10	CCL7		CX3CL1	CMTM3			CKLF		CCR4	CXCL3	CCL2
CXCL2	CCR5		CXCR5	CXCL1			CXCL5		CKLF	CXCL6	CCL24
CXCL5	CCR8		CXCR6	CXCL12			CXCR1		CMTM1	HIF1A	CCL27
CXCR3	CKLF		PPBP	CXCL16			CXCR6		CMTM3		CCL28
GPR17	CMTM3		SLIT2	CXCL2			IL1B		CXCL3		CCL5
IL4	CX3CR1		TNF	CXCL8			PPBP		CXCL6		CCR2
IL16	CXCL12		XCR1	CXCL9			SLIT2		CXCR1		CKLF
	CXCL16			GPR17			TNF		FPR1		CMTM3
				XCL2					TLR4		CMTM4
											CXCL1
											CXCL2
											CXCL3
											CXCL8
											CXCR1
											CXCR6
											GPR17
											HIF1A
											IL16
											PPBP
											SLIT2
											TNF

inflammation [20]. Also, CCR9^{+} T cells from mesenteric lymph nodes of CD patients are enhanced to produce IFN-gamma and IL-17 compared to controls [21], and the therapeutic targeting of CCR9 by antagonists has been proposed [22], although it was not effective as induction therapy in a clinical trial in patients with moderate and severe CD [23]. Secretion of CCL20 by epithelial cells in response to inflammation has been described to attract IL-17 producing lymphoid cells [24], and neutralization of CCL20 reduces T cell recruitment and attenuates colitis in a murine TNBS model [25]. CXCL5 upregulation has also been involved in early stages of IBD, where damage to the epithelial cell layer is still limited [26]. Although these genes are also shown upregulated in healthy and mildly inflamed tissue of CD patients on ustekinumab, a compensatory transcriptional downregulation of chemokines such as CCL17, CCL2, CCL3, and CCL23, involved in T cell chemotaxis, inflammatory monocyte trafficking and macrophage and NK cell migration [27] is also observed, suggesting an earlier regulatory effect of anti-IL12/23 on these genes.

In the case of severely inflamed tissue, all treatments showed a negative regulatory activity over a wide range of chemokines, thereby contributing to explain the reduction in differences between tissues observed in Fig. 3A. Genes that showed an upregulated expression in healthy and mildly inflamed tissue of patients on anti-TNF-α and on anti-integrin anti-α4β7 changed their expression pattern towards a significant downregulatory profile compared to untreated severely inflamed tissue. In the case of adalimumab, a set of genes such as CCL19 or ACKR1, involved in T cell and DC homing to lymph nodes, chemokine scavenging and neutrophil transmigration, stayed upregulated in this tissue condition. CCL27 behaved similarly, which may account for paradoxical anti-TNF-α reactions observed in CD patients such as psoriasis, as CCL27 is involved in recruiting T cells to skin [28]. On the other hand, genes that already showed a downregulatory profile in patients on ustekinumab in healthy and mild inflammation confirmed this tendency in severely inflamed tissue.

The ability of anti-IL12/23 in early chemokine-receptor gene regulation can be noticed by the enrichment analysis with the increased expression of genes implicated in the negative regulation of leukocyte tethering and rolling and of DC apoptosis, such as CCL21 and CXCL12, both in healthy and mildly inflamed tissue. This downregulation in the tissue may be explained by the blockade induced by ustekinumab in Th subpopulations differentiation [29], thus reducing T cell gut homing. This modulation probably leads to the transcriptional reduction in chemokines that activate Th1 cytokine production observed in severely

inflamed tissue. On the contrary, the anti-TNF-alpha immunomodulatory activity of adalimumab is probably exerted by reducing neutrophil migration [30], as observed by the negative regulation of chemokines such as CXCL2 in mildly and severely inflamed tissue, and through a wider interference with the innate immunity. In fact, another important effect of adalimumab, shared with vedolizumab, on severely inflamed tissue is the regulation of DC antigen processing and presentation and the T cell polarity.

Considering this evidence, transcriptional regulation of the chemokine-coding gene set by biologic therapies might require a threshold of inflammatory activity. In that scenario, the transcriptional upregulation observed in healthy and mildly inflamed tissues would be caused by recurrent disease triggers in favorable microenvironments, fostering immune cell recruitment. These cells would further produce mediators fueling inflammation in specific intestinal locations. The increasing inflammatory burden would reach a threshold susceptible of immunomodulation by treatments, as observed in the severely inflamed tissue for all three biologics. Additionally, considering the number of genes that are not modulated by each treatment in healthy and mildly inflamed tissues, it is tempting to speculate that failure in down-regulating sets of chemokine-related genes in specific areas at this early stage of inflammation may contribute to their progress towards a more severely inflamed status. The association of treatments with increased transcriptional activity of chemokines compared with untreated samples shown for healthy and mildly inflamed tissue requires further studies. This increment may be reflecting the significant difference between untreated debuting patients and treated patients with previous flares. It is conceivable that tissue activation in the first group of patients is more moderated compared with that present in the preactivated tissue of patients with a larger disease duration. In fact, altered endothelial cell function is associated with persistent inflammation [31,32].

Finally, we acknowledge that the reduced sample size constitutes a study limitation. However, the neat pattern in which samples were grouped by biologic treatment and inflammatory state and not by patient, the larger effects of these two variables over interindividual variability and the fact the study has a within-subject design regarding inflammatory state support consistency of the results obtained on these contrasts. Also, as an additional consideration, although healthy tissue most likely corresponds to unaffected areas rather than healed tissue (as no signs of inflammation or scars were evidenced in any of the healthy regions collected, either endoscopically or histologically), we cannot discard that this tissue might have been active in the past.

In summary, the chemokine network shows wide transcriptional differences between tissues in active untreated patients. Treatments reduce these differences and homogenize transcriptional activity of chemokine and chemokine receptors among tissues. In severely inflamed tissue, all three biologic monotherapies are associated with a significant regulation of the chemokine gene network. The transcriptional regulation of the chemokine network has distinctive features for each treatment. In addition to their specific mechanisms of action, adalimumab widely modulates processes of the innate immunity; ustekinumab contributes to the control of progression from innate to adaptive responses; and vedolizumab is associated with a reduction of DCs functional activity. Despite treatments-specific differences, there is a shared modulatory effect on chemokines regulating T cell response by all treatments. Nevertheless, to observe such an effect, an inflammatory activity threshold seems necessary. This threshold is reached at different inflammatory level for each treatment.

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CRediT authorship contribution statement

AG, LM: Inclusion and management of patients. **JRA:** endoscopic procedures. **AP:** histologic analysis. **RL, EC:** sample processing and experimental work. **JM:** immunofluorescence microscopy. **AMG, JPLA:** performed statistical analysis of gene expression data. **RF:** study design and manuscript writing. All authors contributed with reviewing and editing of the original draft.

Conflict of interest statement

Authors state no conflict of interests.

Availability of data and material

Raw data related to this study has been deposited in Mendeley Data and is citable and publicly available: Lopez-Atalaya, Jose P.; Francés, Rubén (2021), "BioMnoTrpyCD", Mendeley Data, V1, ⟨https://doi.org/10.17632/s4r6xfdhjs.1⟩.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112653.

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