More fuel to the fire: Some patients with non-celiac self-reported wheat sensitivity exhibit adaptive immunological responses in duodenal mucosa.

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Abstract

Background: In contrast to the well characterized Celiac Disease (CD), the clinical scenarios encompassed by non-celiac self-reported wheat sensitivity might be related to different antigens that trigger distinct immune-inflammatory reactions. Although an increased number of intestinal intraepithelial lymphocytes (IEL) is observed at the inception of both diseases, subsequent immunopathogenic pathways seem to be different. We aimed to describe the cytokine profile showcased by the duodenal mucosa of patients with self-reported wheat sensitivity.

Methods: In a blind, cross-sectional study, we included duodenal biopsies from 15 consecutive untreated patients with active CD, 9 individuals with self-reported NCSRWS and 10 subjects with dyspepsia without CD and food intolerances. Using Immunohistochemistry and flow- cytometry we determined the presence of pro-inflammatory cytokine expressing monocytes and monocyte-derived dendritic cells involved in innate immune activation, cytokine-driven polarization and maintenance of Th1 and Th17/Th 22, and anti-inflammatory/profibrogenic cytokines.

Results: The percentage of cells expressing all tested cytokines in lamina propria and epithelium was higher in CD patients than control group. Cytokines that induce and maintain Th1 and Th17 polarization were higher in CD than in NCSRWS and controls; and higher in NCSRWS compared to controls. Similar differences were detected in the expression of IL-4 and TGF- β1, while IL-10-expressing cells were lower in NCSRWS patients than in controls and CD subjects.

Conclusions : NCSRWS patients exhibit components of both, innate and adaptive immune mechanisms but to a lesser extent compared to CD.

Introduction

Celiac disease (CD) and non-celiac gluten sensitivity (NCGS) are gluten related disorders (GRD) that share clinical characteristics but have marked serological and histological differences. While autoantibodies and duodenal villus atrophy (VA) are mandatory to diagnose CD, they have to be absent in order to establish a presumptive diagnosis of NCGS (1-3).

Due to the lack of specific biomarkers the diagnosis of NCGS is largely based upon clinical characteristics with symptoms improving during a gluten-free diet and symptomatic relapse while on a gluten containing diet, along with normal duodenal biopsies and negative CD serology. In these cases, wheat allergy should also be ruled out (4-6).

CD is a well-characterized disease with specific histological and serological features that affect genetically predisposed individuals. The ingestion of gluten and related proteins triggers well known immunopathogenic mechanisms orchestrated by CD4+ T helper 1 (Th1) and Th17 cells that result in mucosal inflammation and VA (7-12).

In contrast, NCGS is a poorly characterized disorder in which the role of gluten as the main antigen and the pathophysiologic mechanisms responsible for the tissue damage and symptoms development are debatable (13). Many subjects that claim to be intolerant to gluten are unable to associate their symptoms with its ingestion when they are exposed to a double-blind gluten-placebo challenge suggesting the participation of other factors or mechanisms (14). Several randomized double-blind cross-over gluten-placebo controlled studies have shown that symptoms in these patients are associated to fermentable short-chain carbohydrates (FODMAP) rather than to gluten (15-17). On the other hand, an experimental model showed that amylase-trypsin inhibitors (ATI) (a group of proteins present in wheat) induce an immune-inflammatory reaction mainly mediated by components of innate immunity in the duodenal mucosa (18). Based on these findings, some authors suggest identifying this entity as non-celiac wheat sensitivity (NCWS) instead NCGS (19). This term includes other potential antigens present in wheat, although excludes, at least from a semantic point of view, the pathogenic role of other cereals. On the other hand, the diagnosis of NCGS must be established by means of complicated double-blind gluten-placebo challenge rarely performed in the clinical setting (6). Thus, in the real world it is quit probably that most non-celiac self-reported gluten intolerant subjects are in fact wheat intolerant rather than gluten intolerant.

The response to FODMAP exhibited by subjects with NCWS is that of food intolerance, lacking an inflammatory component and histopathological alterations. In contrast, exposure to ATI exert an immune mediated reaction that could explain the symptoms and the histopathological alterations observed in some NCSRWS patients (18,20).

Thus, NCSRWS may encompass different clinical and pathogenic scenarios: 1. A kind of food intolerance induced by FODMAP, 2. An immune-inflammatory reaction trigger by ATI mediated by innate immunity components and, 3. A true sensitivity to gluten in which current evidence sustains a paramount role of innate immunity, although according to some authors, certain components of adaptive immunity may be involved (21-23).

In this exploratory study we aimed to describe the cytokine profile and quantify the pro-inflammatory cytokine-expressing monocyte and monocyte-derived dendritic cells involved in innate immune activation, cytokine-driven polarization and maintenance of Th1 polarization and Th17/Th22, and anti-inflammatory/profibrogenic cytokines in the duodenal mucosa of a group of subjects with self-reported wheat sensitivity. To our knowledge, this is the first depiction of cytokine production on duodenal mucosa of NCSRWS patients by immunohistochemistry, since other studies have evaluated cytokine levels on serum and peripheral blood mononuclear cells or duodenal mucosa culture supernatants.

Methods

Patients and controls

In this blind, cross-sectional study we included 15 consecutive untreated patients with active CD and 9 individuals with NCSRWS who attended to the Department of Gastroenterology at the Instituto Nacional

de Ciencias Médicas y Nutrición Salvador Zubirán, a tertiary referral medical facility in Mexico City. All patients were enrolled over a period of 2 years (2014-2016).

CD was diagnosed when patients met the following criteria: 1) Compatible clinical data: chronic diarrhea, weight loss, bloating, abdominal discomfort, fatigue or nutrient deficiencies, 2) positive anti-endomysium (EmA IgG/IgA; IF, Inova Diagnostics, San Diego, CA, USA. Normal < 1:5), anti-transglutaminase IgA (anti-tTg IgA; ELISA, Orgentec; Mainz, Germany. Normal < 10 U/mL) and anti-deamidated gliadin peptide antibodies (DGP IgA and IgG; ELISA, Orgentec; Mainz, Germany. Normal < 10 U/mL) and, 3) VA according to Marsh-Oberhuber criteria (24).

The diagnosis of NCSRWS was considered when patients presented with: 1) intestinal and extra intestinal symptoms associated with the ingestion of gluten-containing food, 2) a clear clinical response while they had a gluten-free diet, 3) relapse of symptoms with the ingestion of gluten-containing foods, 4) negative serological markers for CD (EmA IgA, anti-tTG IgA, DGP) and wheat allergy (IgE serological test) and, 5) normal duodenal biopsies.

Only NCSRWS patients had a challenge with gluten free/gluten containing diet. All of them were evaluated by a qualified nutritionist with expertise in CD. Symptoms severity (abdominal discomfort or pain, bloating, diarrhea, and constipation) was assessed at baseline, while on an unrestricted diet, 6 weeks after following a gluten-free diet and after completing a 6 weeks challenge with 10 grams of gluten per day using a visual analog scale (VAS; 0-10).

Diet compliance was evaluated in out-patient visits scheduled every 2 weeks during the gluten-challenge. All serological tests (EmA IgA, anti-tTG IgA, DGP) were performed at baseline visit and after completion of the gluten challenge. Headache, tingling or numbness in feet or hands, fatigue, musculoskeletal pain, foggy mind, rash and oral ulcers were considered extra-intestinal symptoms and they were specifically evaluated using VAS. We considered a good clinical response to the gluten-free diet when there was a decrease in the intensity of symptoms of at least 50% compared to the baseline VAS. We did not perform a double-blind gluten/placebo-controlled trial challenge in any case.

We excluded patients with other gastrointestinal diseases, history of gastrointestinal surgery, active or previous infection diseases, clotting disorders, renal insufficiency, pregnancy or breast feeding, active use of antimicrobial, probiotics, immunosuppressive drugs, non-steroidal anti- inflammatory drugs or corticosteroids.

The control group was composed by 10 subjects that fulfilled ROMA III criteria for functional dyspepsia who had undergone an upper endoscopy with both, negative CD serologies and normal duodenal histology (25). None of them underwent a gluten containing/gluten free challenge.

Biopsy Sampling

Duodenal biopsy was performed in all cases while patients had on a regular gluten containing diet.

During upper endoscopy four tissue samples from the second portion of the duodenum were obtained; two of them were placed immediately in ice-chilled Hank buffer solution (HBSS) /5% fetal bovine serum (SFB, GIBCO). The others were fixed in 10% formaldehyde and subsequently embedded in paraffin wax and cut into sections 4 µm thick.

Mucosal Lymphocytes (mLs) (IELs) Isolation from Duodenal Tissue

Mucosa samples (epithelium and lamina propria) were cut with a scalpel blade and incubated in phosphate buffer 1x (PBS) / ethylenediamine tetra acetic acid (EDTA) 2mM at 34ºC for 30 min while being agitated. After that, samples were treated with Collagenase IV (Sigma) at 60 U/ml for 1 h at 34°C while being agitated. The cell suspension was then passed through a 40µm cell strainer (Cell Strainer BD Falcon), washed with 2 ml of PBS, and centrifuged at 800 g for 10 min at 25°C. The resulting pellet was homogenized in 1 ml of PBS and incubated with 1 µL of Brefeldin A (BD Golgi Plug) for 1 h at 37°C with 5% CO2. Live-dead assay and cellular count was realized on cellular samples (>90%) on Neubauer chamber (trypan blue) (26)

Immunohistochemistry

Tissues placed on positively charged slides were incubated with mouse monoclonal anti-human IL-1β, IL-6, IL-8, IL-10, IL-15, IL-22, IL-23, IFN-γ, TNF-α, and with rabbit polyclonal anti-human IL-2, IL-12p40, IL-17A, IL-21, or TGF-β1 antibody (Abcam, Cambridge, MA, USA) or anti-human IL-4 antibody (Bio Legend Inc., San Diego, CA, USA) at 10 µg/mL during 30 min. Binding was detected with Universal Dako labelled streptavidin biotin reagent+peroxidase for primary antibodies from rabbit, mouse and goat (Dako, Glostrup, Denmark). Spleen and ganglion samples were used as a positive control. Negative controls were carried out with normal human serum (1:100) and with the IHC universal negative control reagent (Enzo Life Sciences, Inc., Farmingdale, NY, USA), while phosphate buffer saline-egg albumin (SIGMA-Aldrich) was use in the reactive blank. Controls excluded nonspecific staining or endogenous enzymatic activities. We examined three different sections of each biopsy. Cytokine-expressing cells were reported as the percentage of positive cells in three fields (X320) taken from the epithelium and lamina propria. Results are expressed as the median, mean and $5^{th}/95^{th}$ percentiles (27)

Peripheral Blood Mononuclear Cells (PBMCs) Isolation

We collected a sample of venous blood to isolate PBMCs by gradient centrifugation on Ficoll-Paque (Merck-Millipore). The bottom was resuspended in 1 mL of PBS 1x /Brefeldin A (BD GolgiPlug) and incubated at 37ºC in 5% CO_2 during 1h. Live-dead assay (trypan blue) and cellular count was realized on cellular samples (>90%).

Flow Cytometry

1X105 PBMCs or mLs were labeled with 5 μL of antihuman CD4-FITC-labeled, monoclonal antibody (BioLegend San Diego, CA). Cells were permeabilized with 200 μL of cytox/cytoperm solution (BD

Biosciences). Intracellular staining was performed with an anti-human Foxp3-PE-, IFN-γ-APC-Cy7-, IL-17A-PE-Cy7- (BioLegend), T-bet-PerCP-Cy5.5- (BD Pharmingen, San Jose, CA), and ROR-γt-APC-labeled (R&D Systems, Minneapolis, MN) mouse monoclonal antibodies. From the electronic bi-parametric gate of the singlets and living cells, we performed an analysis in the CD4+ lymphocytes population to identify CD4+/Foxp3+ cells, CD4+/T-bet cells, CD4+/INF-γ cells, CD4+/ROR-γt+ cells, CD4+/IL-17A cells. Results are expressed as the relative percentage of CD4+/IL-17A+−, CD4+/IFN-γ+−, CD4+/Foxp3+−, CD4+/T-bet+−, and CD4+/ROR-γt+−expressing cells in each gate. For an autofluorescence control, we ran an unstained and permeabilized cell sample. An AbC anti-mouse bead kit (Invitrogen, UK) was used to adjust instrument settings, to set fluorescence compensation, and to check instrument sensitivity. Fluorescence minus one (FMO) controls were stained in parallel. Samples were analyzed with an Attune Acoustic Focusing Cytometer Blue/Red (Life Technologies). We recorded more than 10,000 events for each sample, and they were analyzed with Attune® Cytometric Software v2.1 (26,28)

Ethical Considerations

This work was performed according to the principles expressed in the Declaration of Helsinki. The study was reviewed and approved by the institutional ethics and research committee (GAS-1298-14/15-1; August 11, 2014). Each patient gave and signed a written informed consent.

Statistical analysis

Due to the exploratory nature of the study we included a convenience sample of consecutive patients with CD, NCSWS and FD. Statistical analysis was performed using GraphPad Prism for Windows (version 6.01 GraphPad software Inc. USA). Immunohistochemical data are expressed as the median, mean and $5^{th}/95^{th}$ percentiles. We used Kruskall Wallis test for non-parametric variables. We performed one-way analysis of variance on ranks by Holm-Sidak method and Dunn's test for all pairwise multiple comparison procedures and comparisons versus a control group. $P \leq 0.05$ was considered statistically significant.

Results

Demographic and clinical characteristics of patients are summarized in Table 1 and Table 2. Diarrhea, abdominal pain and bloating were the most frequent symptoms in both, CD and NCSRWS patients.

All NCSRWS patients completed the challenge. While on the gluten challenge, they experienced bloating and abdominal pain and 1 of them presented oral itching; in all cases symptoms improved with the gluten-free diet.

One patient with NCSRWS (DGP IgG 63 U/mL) and one in the control group (DGP IgA 36 U/mL) had titers of anti-DGP above the upper limit of normal, but none of them showed alterations neither in serology nor histology.

Percentage of Peripheral CD4+ T cell subpopulations in PBMCs

No differences were observed in the number of CD4+ T cells (Figure 1A), CD4+/Foxp3+ (Figure 1C), CD4+/T-bet+ (Figure 1E), CD4+/IFN-γ+ (Figure 1G), CD4+/ROR-γt+ neither CD4+/IL-17A+ cells (Figure 1K) amongst the groups.

Percentage of mucosal subpopulations in duodenal tissue

The percentage of CD4+/mLs was higher in CD patients compared to control group (P=0.003, Figure 1B). No differences were found between CD patients and NCSRWS or between NCSRWS and the control group.

CD patients had a significantly higher CD4/FoxP3 percentage in duodenum compared to the control group. (P=0.039, Figure 1D). The NCSRWS CD4/FoxP3 percentage was similar to both, CD and control group.

T-bet and ROR-γt+ were higher in CD patients versus control group (P=0.036, Figure 1F and P=0.03, Figure 1J, respectively). No differences were observed between CD patients and NCSRWS. Neither there was any difference when comparing NCSRWS to the control group. The percentage of IFN-γ +− and IL-17A−expressing CD4 mucosal T cells in the CD group, although higher than NCSRWS, was not statistically significant amongst the three groups (Figure 1H and 1L).

Pro-inflammatory cytokines in duodenal tissue

The percentage of IL- β− and TNF-α−expressing cells in tissue of CD and NCSRWS patients was significantly higher compared to the control group. Tissue of NCSRWS patients had statistically significant lower levels of IL-1 β− and TNF-α−expressing cells compared with CD patients (Figure 2A,B).

The number of IL-6− and IL-8−producing cells was significantly higher in CD patients compared with control group and NCSRWS patients. No differences in the number of IL-6+ or IL-8+ cells were found in the NCSRWS group compared to the control group (Figure 2C,D).

Cytokines involved in the differentiation and maintenance of Th1 in duodenal tissue

The percentage of IL-2+ and IFN- γ+ cells of CD patients was higher compared to NCSRWS and control group. No statistically significant differences in the number of IL-2+ or IFN- γ + cells were determined in the NCSRWS group compared to the control group (Figure 3A,D).

The IL-12 and IL-15 cell percentage of CD patients was conspicuously higher when compared to the control group and NCSRWS. The number of IL-12− and IL-15−expressing cells was significant higher in NCSRWS compared to control (Figure 3B,C).

Cytokines involved in the differentiation and maintenance of Th17/Th22 in duodenal tissue

The percentage of IL-17A+, IL-21+, IL-22+ and IL-23+ cells of CD patients was higher versus control group, and NCSRWS patients (Figure 4A-D). Tissue of NCSRWS group had statistically significant lower levels of

IL-17A−, IL-21−, IL-22− and IL-23+ −expressing cells compared with CD patients (Figure 4A-D).

Anti-inflammatory/Profibrogenic cytokine expression in duodenal

No differences were observed in IL-4 cell percentage when compared CD patients or NCSRWS versus control group (Figure 5A).

TGF-β1− and IL-10−expressing cells from CD or NCSRWS patients were higher versus control group (Figure 6 B-C). No statistically significant difference was found between CD and NCSRWS patients.

Discussion

CD is a well-characterized disease with specific histological and serological features and established immuno-pathological mechanisms triggered by the ingestion of gluten and related proteins in genetically predisposed individuals (9-12). In contrast NCGS is a disorder seeking its own identity. It is a condition that encompasses different clinical scenarios including subjects with irritable bowel syndrome (IBS) and patients with food intolerances that experience abdominal or extra-intestinal symptoms after consumption of gluten; typically, they improve with a gluten-free diet (19,29,30).

The ambiguity about its existence is based on the absence of specific biomarkers and histological characteristics. Its diagnosis has relied on the clinical response observed during complicated double-blind gluten-placebo controlled challenges that have used different vehicles and doses of gluten (5,14,31).

In an attempt to standardize NCGS diagnosis, a group of experts met in Salerno and proposed a double-blind placebo-controlled challenge using 8 grams of gluten administered over 2 periods of one week separated by one-week wash-up term (6).

Since the Salerno criteria is difficult to fulfill in the clinical setting, once CD and wheat allergy have been reasonably ruled out, the presumptive diagnosis of NCGS is based merely on the clinical response to a diet with and without gluten. However, even with double-blind gluten-placebo studies a large number of self-defined gluten intolerant subjects are unable to relate gluten ingestion to their symptoms, raising the possibility that other cereals components, such as FODMAP and ATI rather than gluten, play the central pathological role (13-18)

Despite these diagnostic caveats, we are confident that our self-considered gluten intolerant patients have wheat sensitivity considering that all of them improved while following a gluten-free diet and relapsed when they were exposed to a gluten-containing diet. On the other hand, CD and wheat allergy were reasonable excluded based on serological test and histological features. Since we did not perform a double-blind gluten-placebo-controlled challenge the participation of other antigens, besides gluten presents in wheat cannot be ruled out. However, in the real world all of these patients are classified as NCGS subjects.

Flow cytometry from all cases during an unrestricted diet showed that CD4+ cell subpopulations were quite different. As expected, CD patients exhibited a wide range of innate and adaptive immune responses when compared to the control group. The percentage of inflammatory and regulatory cells CD4+ T, CD4+/Foxp3+, CD4+/T-bet+, CD4+/ROR-γt+ were higher in CD than in control group and NCSRWS patients. Importantly, the percentage of inflammatory and regulatory CD4+ cells was higher in the group of NCSRWS patients when compared to the control group but lower than that observed in CD subjects. Although this finding did not reach a statistical significance, it is similar to previous reports suggesting that an inflammatory process is present in these self-reported wheat intolerant subjects (19).

Immunohistochemical analysis showed the most noticeable changes. The percentage of pro-inflammatory cytokine-expressing cells in the duodenal mucosa was higher in patients than in controls. The inflammatory response was conspicuously higher in CD subjects although pro-inflammatory cytokine-expressing cells were also evident in NCSRWS patients, except for IL-6- and IL-8-expressing cells. These findings have been previously reported in sera. In fact, to our knowledge, this is the first depiction of cytokine production on duodenal mucosa from NCSRGS patients by immunohistochemistry, since other studies have evaluated cytokine levels on serum and peripheral blood mononuclear cells or duodenal mucosa culture supernatants by ELISA (32-36).

Present evidence suggests that innate immune response plays a central role in the pathophysiology of NCGS. Increased expression of toll-like receptor 2 (TLR-2) and 4 (TLR-4), claudin 4 (CLD-4), and TNF-α has been shown by different groups in subjects with self-reported gluten intolerance (37). According to this concept, IL-1 and TNF-α-expressing cells were higher in NCSRWS patients compared to the control group. These findings support the widely demonstrated participation of innate immunity in both, CD and NCGS (10,11,14,35).

Since our objective was to determine expression of cytokine between CD and NCSRWS patients we did not consider evaluating TLRs. It should be noted that our results about IL-6 and IL-8 in NCSRWS patients are quite similar to those reported in mononuclear cells culture supernatants (ELISA) from NCGS subjects (32). On the other hand, we determined the presence of IL-6+ and IL-8+ cells in the tissue by immunohistochemistry, but not the amount of cytokine that each cell produces .

Th cell polarization from naïve precursors is a tightly controlled process where IL-12 and IL-15 play a central role as factors involved in the differentiation of Th1 response (38,39).The secreted IL-2 by activated antigen-specific CD4+ and CD8+T cells, is consumed at the same and distant sites by cells expressing the IL-2Rα (effector T cells, NK cells and Tregs). IL-2 acts via STAT5 and influence the differentiation of Th1, Th2 and Th17 cell subsets, also it is important to maintain the transcriptional program for Treg function (40).

In addition, IL-2 probably stimulates the differentiation of other cell groups while IL-12 does it for Th1 and, IL-22 and IL-23 for Th17. In contrast, IL-15 not only promotes the increase of IEL's in CD but also supports Th1 and Th17 response (41-43). Meeting these concepts, we observed that the percentage of cytokine expressing cells that induce and maintain Th1 and Th17 polarization in the mucosa of CD

patients was higher compared to the other groups. Interestingly, IL-2 and IL-12 expressing cells were also increased in NCSRWS group compared to the control group, suggesting the participation of some adaptive immunity components in these self-reported wheat intolerant subjects.

While IL-21, a member of the common gamma chain (gc) family of cytokines is expressed by multiple immune cell types including activated CD4+T cells, T follicular helper cells and NK cells, IL-17 is produced not only by CD4+ T lymphocytes, but also by innate immune cells such as γδ T, neutrophils, macrophages, NK, NKT cells and non-immune cells including epithelial and parenchymal cells, which constitute the first line of host defense, acting before adaptive immunity can be initiated, thus, it is not so clear whether the main role of IL-17 is on the innate or the adaptive immune response (44,45).

Recent evidence in NCGS patients also supports the participation of adaptive immune mechanisms. High levels of IFN-g have been found in the duodenal and rectal mucosa of NCGS patients challenged with gluten (46, 47). Moreover, it has been shown that these subjects are able to produce specific antibodies to native gliadins and, at least 50% of them compared to 30% of the general population, express HLA-DQ2/DQ8, haplotypes the risk for CD (48).

It should be noted that we found haplotypes of risk for CD in 6 of 8 patients with NCSRWS while all CD subjects had HLA DQ2/DQ8 genes.

TGF-β1-expressing cells were notably higher in both, CD and NCSRWS patients. It is mostly certain that this finding is due to an attempt to control the inflammatory process elicited by peptides from prolamins in the case of CD or other antigens recognize in NCSRWS patients (18,20). Remarkably IL-10-expressing cells were not increased neither in CD nor in NCSRWS. In fact, they were lower in NCSRWS than in the other groups.

Our findings in CD patients are quite similar to those widely reported in the literature but some differences can be noted in our wheat intolerant patients. At the present time self-reported gluten-intolerant subjects cover a wide range of unspecific clinical scenario. Our patients with NCSRWS were diagnosed on clinical bases excluding wheat allergy and CD, however, we did not perform a double-blind gluten-placebo challenge in any case and the participation of other antigens, besides gluten, could not be excluded. It could be argued that in fact, these could be patients with seronegative CD because the majority had haplotypes of risk (HLA DQ2/DQ8 genes) but all of them showed a normal duodenal mucosa while they were on a gluten-containing diet (48).

Despite these flaws and limitations on regards the diagnosis of NCGS, these patients represent a true challenge in clinical practice. At the present time it seems clear that other wheat components like FODMAP or ATI are able to trigger

symptoms and immune-inflammatory reactions in some non-celiac self-reported wheat intolerant subjects.

Conclusions

Non-celiac self-reported wheat sensitive subjects show components of both, innate and adaptive immunity response in the duodenal mucosa . We believe that our results provide one more piece in the complicated puzzle of wheat sensitivity.

Abbreviations

CD. Celiac disease

NCGS. Non-celiac gluten sensitivity

NCSRWS. Non-celiac self-reported wheat sensitivity

GT-FD. Gluten tolerant patients with functional dyspepsia

IL-4. Interleukin 4

TGF- β1. Tumor growth factor β1

IL 10. Interleukin 10

HLA. Human leukocyte antigen

GRD. Gluten related disorders

VA. Villous atrophy

Th1. T helper 1 lymphocytes

Th17. T helper 17 lymphocytes

Th22. T helper 22 lymphocytes

EmA IgA. Anti-endomysium antibodies

Anti-tTg IgA. Anti-transglutaminase antibodies

AGA-DGP IgA and IgG. Anti-deamidated gliadin peptide antibodies

GFD. Gluten free diet.

VAS. Visual analog scale

FD. Functional dyspepsia

HBSS. Hank buffer solution

SFB. Fetal bovine serum

IEL. Intraepithelial lymphocytes

mLs. Mucosal lymphocytes

PBS. Phosphate buffer

EDTA. Ethylenediamine tetra acetic acid

IL-1β. Interleukin 1 β

IL-6. Interleukin 6

IL-8. Interleukin 8

IL-15. Interleukin 15

IL-22. Interleukin 22

IL-23. Interleukin 23

IFN-γ. Interferon γ

TNF-α. Tumor necrosis factor α

IL-2. Interleukin 2

IL-12p40. Interleukin 12p40

IL-17A. Interleukin 17A

IL-21. Interleukin 21

PBMC. Peripheral blood mononuclear cells

ULN. Upper limit of normal

IBS. Irritable bowel syndrome

TLR-2. Toll-like receptor 2

TLR-4. Toll-like receptor 4

CLD-4. Claudin 4

NK. Natural killer cells

Treg. T regulatory cells

FODMAP. Fermentable short-chain carbohydrates

ATI. Amylase-trypsin inhibitors

Declarations

Ethics approval and consent to participate

The protocol was approved by Research and Ethics Committees of Instituto Nacional de la Nutrición Salvador Zubirán (GAS-1298-14/15-1; August 11, 2014). Each patient signed a written informed consent to participate.

Consent for publication

“Not applicable”

Availability of data and materials

The datasets during or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

None to report.

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Author´s contributions

A.I.C.-R., and L.U. conceived and designed experiments. J.F.-C. Performed experiments. J.C. recruited the patients and controls. A.I.C.-R., L.U., J.F.-C., M.P., Y.L.-V- and J.C.-G. Analyzed data. A.I.C.-R., M.P, L.U., and J.F.-C. Wrote and edited the manuscript.

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CONFLICTS OF INTEREST
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Specific author contributions: A.I.C.-R., and L.U. conceived and designed experiments. J.F.-C., and R.M.-C. Performed experiments. J.C.-G. recruited the patients and controls. A.I.C.-R., L.U., J.F.-C., R.M.-C., Y.L.-V- and J.C.-G. Analyzed data. A.I.C.-R., L.U., and J.F.-C. Wrote and edited the manuscript. Financial support: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.
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Tables

Table 1. Main clinical characteristics of patients with celiac disease (CD), non-celiac gluten sensitivity (NCSRWS) and control.

	CD (n=15)	NCSRWS (n=9)	Control (n=10)
Age (years)			
Mean ± SD	55.2 ± 15.9	49.8 ± 13.6	53.2 ± 9.1
Female	13	9	5
Food allergies	2 *	1**	0
Autoimmune disease	12	2	4
Osteopenia/osteoporosis	5	3	1
Body mass index Kg/m^2			
Mean ± SD	23.4 (21-39)	21.5 (18-25)	25 (22-28)

* Fish, ** Berries

Table 2. Serological markers (antibodies values are expressed as median with min-max) and the main laboratory variables (chemistries are expressed as the number of patients with abnormal values).

	CD (n=15)	NCSRWS (n=9)	Control (n=10)
DGP IgA (U/mL)	51 (12.3-132.3)	4.4 (3.5-19.6)	5.5 (4.2-36.6)
DGP IgG (U/mL)	32 (4-143)	6.2 (3.9-63.1)	4.2 (3.2-9.5)
tTg IgA (U/mL)	17 (2.3-436.1)	3.1 (0.6-15)	2.5 (1.5-4.9)
EmA IgA positive	11 (73%)	0	0
HLA DQ2/DQ8 positive	12* (100%)	6** (75%)	NA
Hemoglobin < 13.0 g/dL	5 (33%)	0	0
Ferritin < 11 ng/mL	3 (20%)	2 (22%)	0
Vitamin B12 < 180 pg/mL	1 (6%)	1 (11%)	NA
Folates < 5.9 ng/mL	6 (40%)	1 (11%)	NA
Vitamin D < 29 ng/mL	11 (73%)	3 (33%)	NA
Vitamin D < 20 ng/mL	5 (33%)	2 (22%)	NA
Albumin< 3.5 g/L	1 (6%)	1 (11%)	0 (0.0)

DGP IgA = IgA anti-deamidated gliadin antibodies DGP IgG= IgG anti-deamidated gliadin antibodies tTg IgA= IgA anti-transglutaminase antibodies EmA IgA= IgA anti-endomysium antibodies. NA= not-available

** HLA performed in 12 patients. ** HLA performed in 8 patients.*

Figures

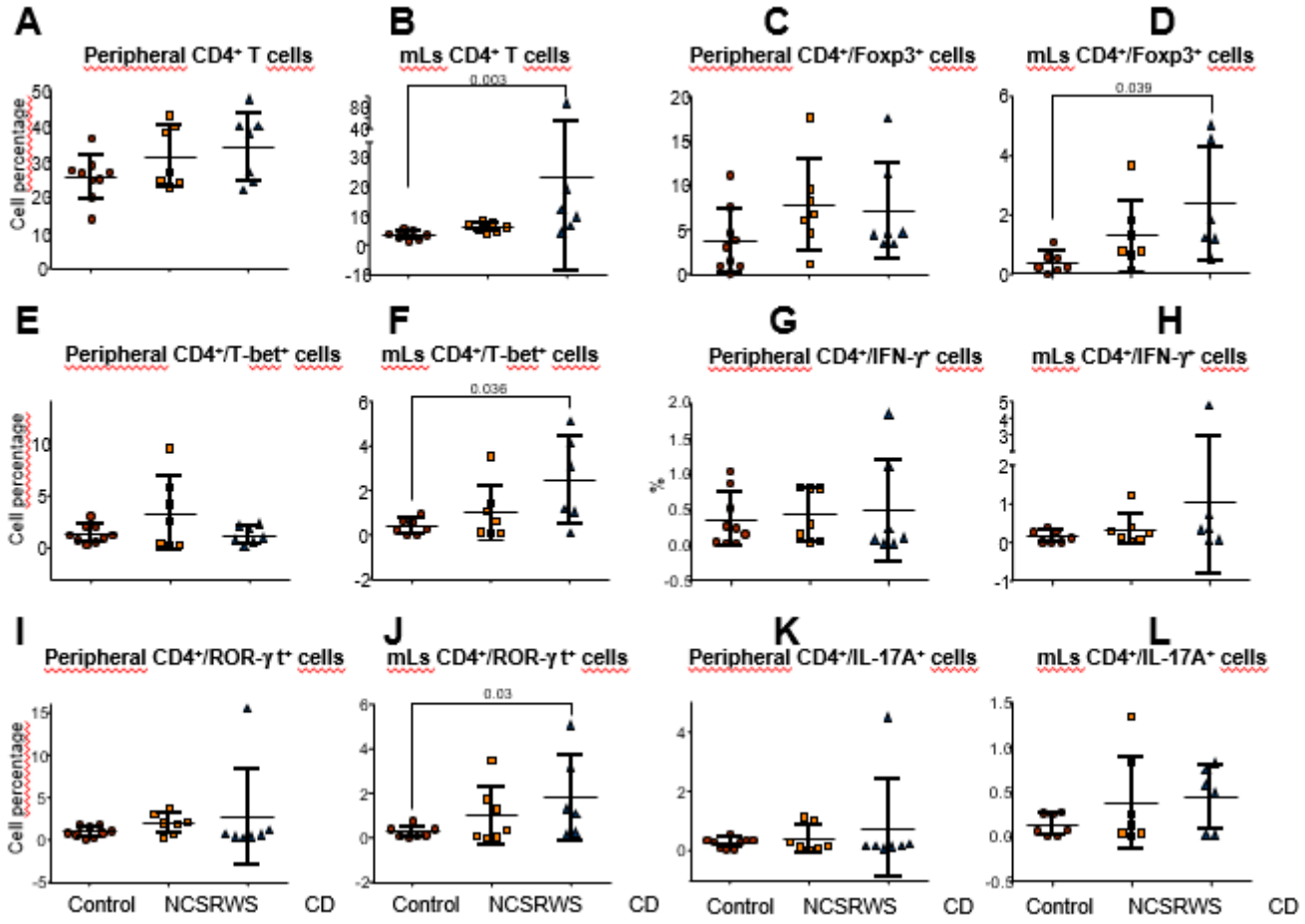

Figure 1

Circulating and mucosal lymphocytes (mLs) in control, non-celiac self-reported gluten sensitive (NCSRGS) and celiac disease (CD) groups. (A) Peripheral and (B) mLs CD4+ T cells; (C) Peripheral and (D) mLs CD4+/Foxp3+ Tregs; (E) Peripheral and (F) mLs CD4+/T-bet+ cells; (G) Peripheral and (H) mLs CD4+/IFN-γ+ cells; (I) Peripheral and (J) mLs CD4+/ROR-γt+ cells; (K) Peripheral and (L) mLsCD4+/IL-17A+ cells. Results are expressed as mean (black line) ± standard deviation. Control: n = 9, NCSRWS: n = 7 and CD: n = 7.

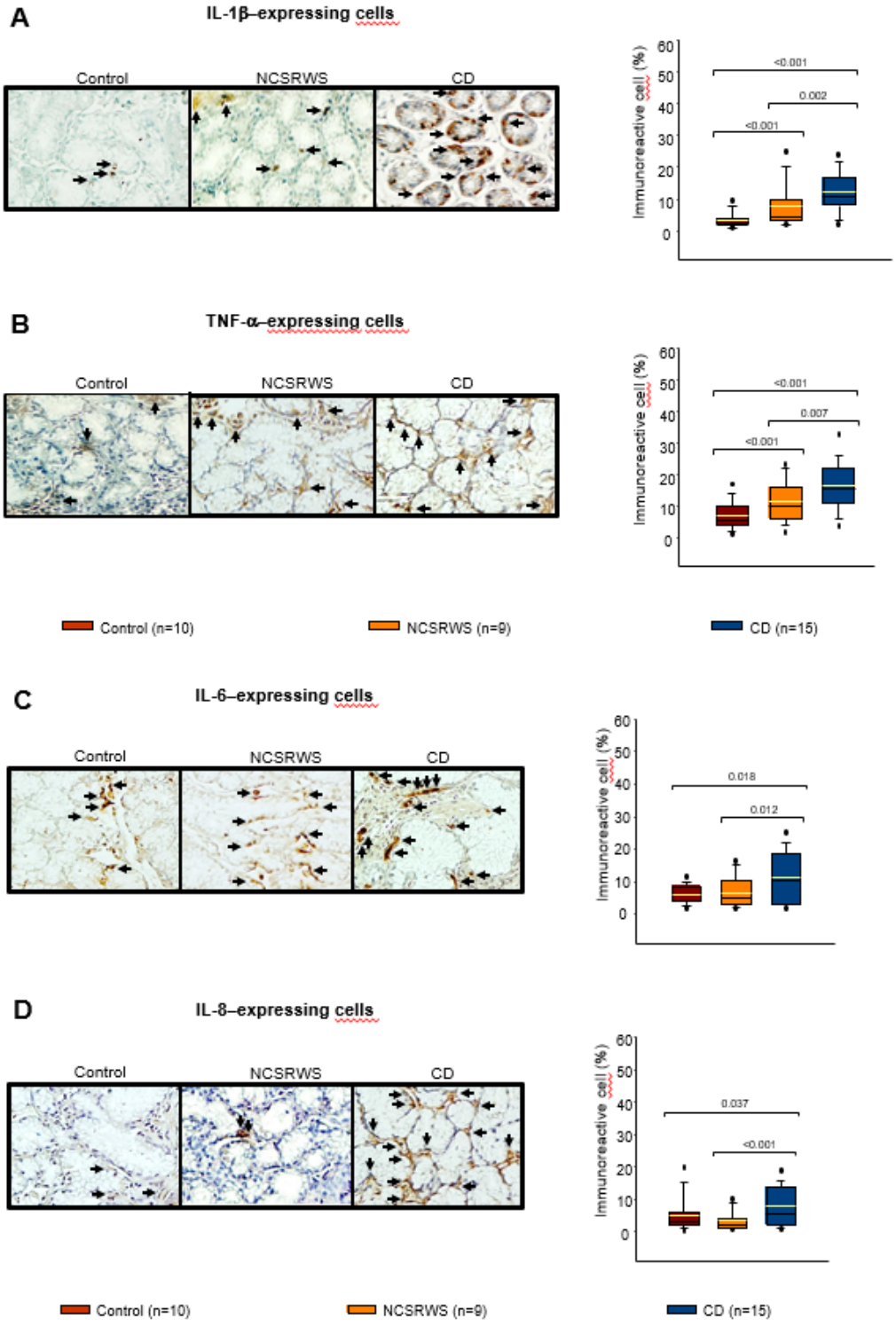

Figure 2

Pro-inflammatory cytokine-expressing cells in Celiac Disease. (A, B, C, and D left panel): Representative immunoperoxidase photomicrographs of control, non-celiac self-reported gluten sensitivity (NCSRGS) and celiac disease (CD). Arrows depict (A) IL-1β, (B) TNF-α, (C) IL-6 and (D) IL-8 immunoreactive cells. Original magnification was x320. (A, B, C and D, right panel): Relative percentage expression of (A) IL-1β,

(B) TNF-α, (C) IL-6 and (D) IL-8. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.

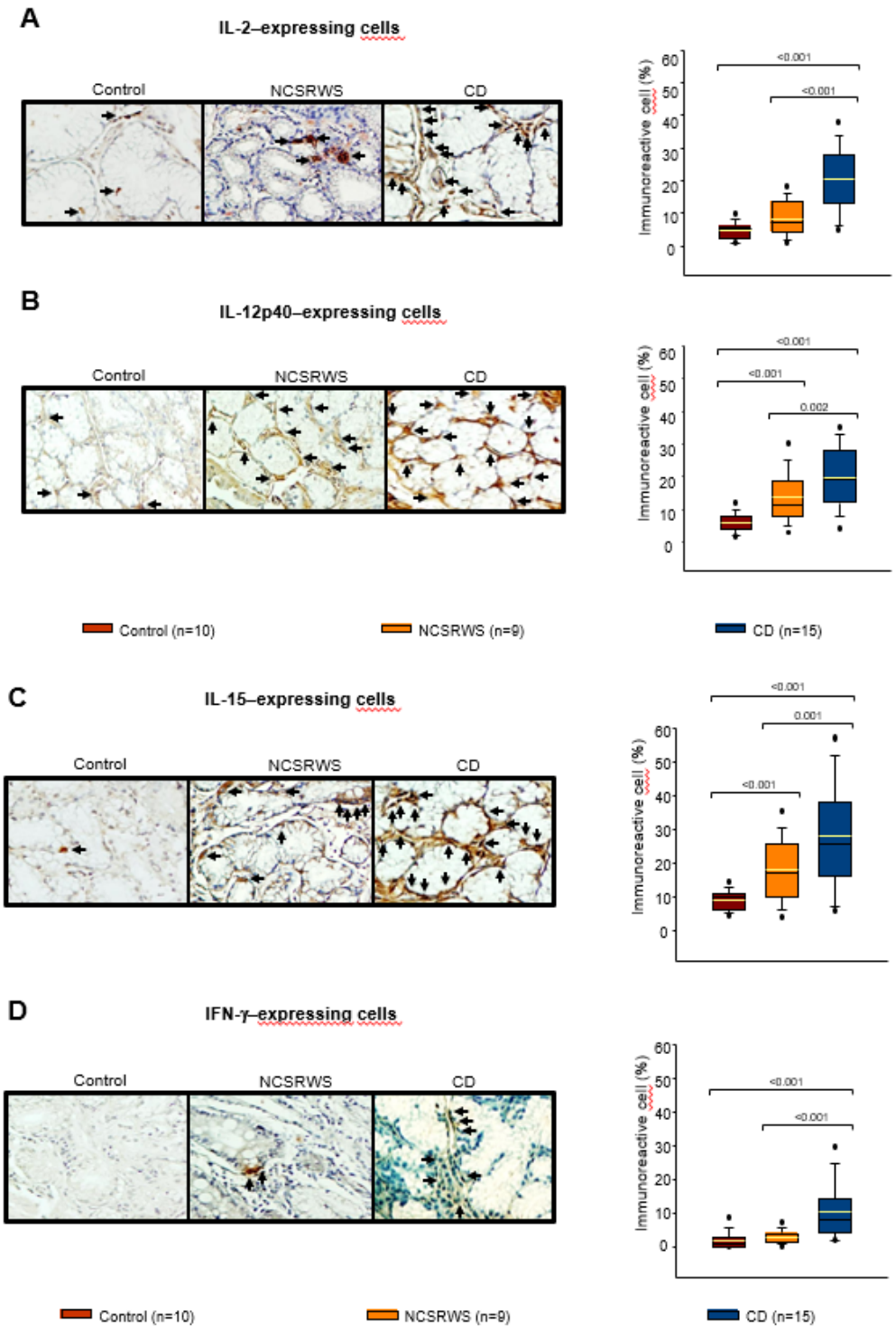

Figure 3

Cytokines that induce and maintain Th1 polarization. (A, B, C, and D left panel): Representative immunoperoxidase photomicrographs of control, non-celiac self-reported gluten sensitivity (NCSRGS) and celiac disease (CD). Arrows depict (A) IL-2, (B) IL-12p40, (C) IL-15 and (D) IFN-γ immunoreactive cells.

Original magnification was x320. (A, B, C, and D, right panel): Relative percentage expression of (a) IL-2, (b) IL-12p40, (c) IL-15 and (d) IFN-. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.

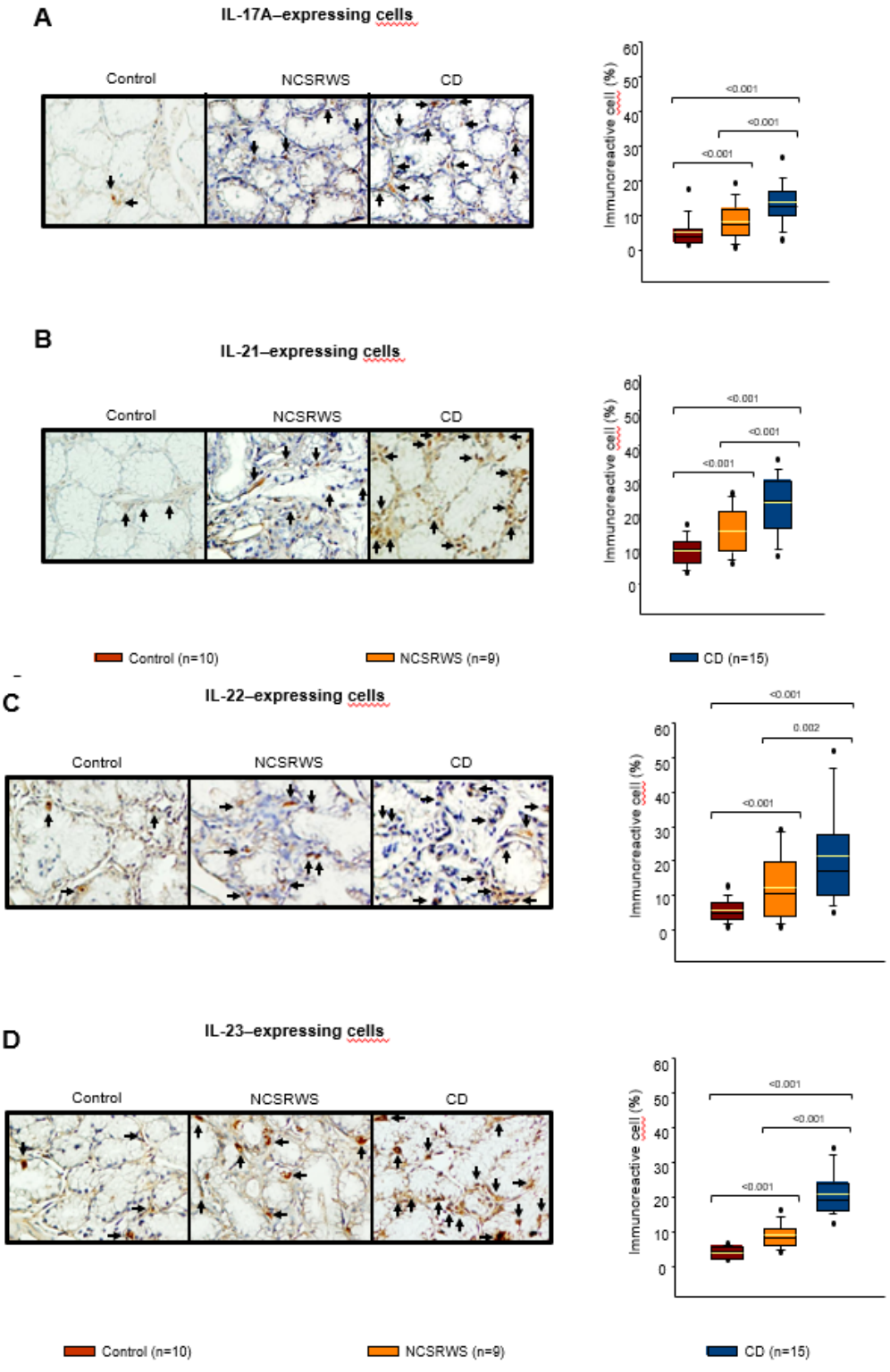

Figure 4

Cytokines that induce and maintain Th17 polarization. (A, B, C, and D left panel): Representative immunoperoxidase photomicrographs of control, non-celiac self-reported gluten sensitivity (NCSRGS)

and celiac disease (CD). Arrows depict (A) IL-17A, (B) IL-21, (C) IL-22 and (D) IL-23 immunoreactive cells. Original magnification was x320. (A, B, C, and D, right panel): Relative percentage expression of (A) IL-17A, (B) IL-21, (C) IL-22 and (D) IL-23. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.

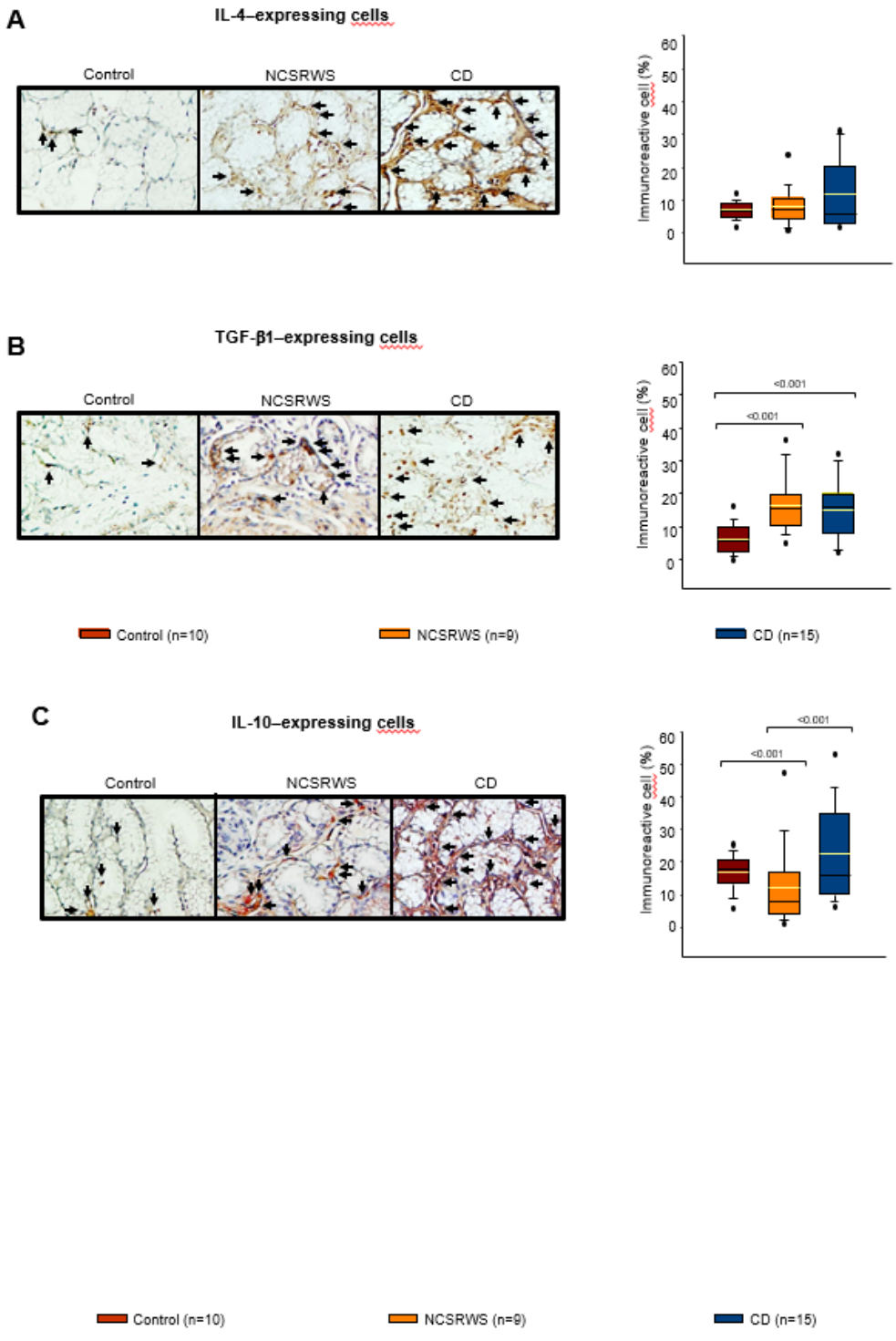

Figure 5

Anti-inflammatory/pro-fibrogenic cytokines. (A, B, C, and D left panel): Representative immunoperoxidase photomicrographs of control, non-celiac self-reported gluten sensitivity (NCSRGS) and celiac disease (CD). Arrows depict (A) IL-4, (B) TGF-β1, and (c) IL-10 immunoreactive cells. Original magnification was x320. (A, B, C, and D, right panel): Relative percentage expression of (A) IL-4, (B) TGF-β1, and (C) IL-10. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.

Supplementary Files

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