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RESEARCH ARTICLE | *Inflammation, Immunity, Fibrosis, and Infection*

TNF-α, IL-17, and IL-22 production in the rectal mucosa of nonceliac wheat sensitivity patients: role of adaptive immunity

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Mansueto P, Di Liberto D, Fayer F, Soresi M, Geraci G, Giannone AG, Seidita A, D'Alcamo A, La Blasca F, Lo Pizzo M, Florena AM, Dieli F, Carroccio A. TNF-α, IL-17, and IL-22 production in the rectal mucosa of nonceliac wheat sensitivity patients: role of adaptive immunity. *Am J Physiol Gastrointest Liver Physiol* 319: G281–G288, 2020. First published July 13, 2020; doi: 10.1152/ajpgi.00104.2020.—In recent years, a new gluten- or wheat-related disease has emerged, a condition labeled "nonceliac gluten sensitivity" (NCGS) or "nonceliac wheat sensitivity" (NCWS). NCWS pathogenesis is still uncertain and attributed to very different mechanisms. We aimed to study the different T-lymphocyte subsets in the rectal mucosa of NCWS patients to demonstrate the possible contribution of adaptative immune response. Twelve patients (11 women, 1 man, age range 23–61 yr, median 32 yr) with a definitive diagnosis of NCWS were recruited at random for the present study. They underwent rectal endoscopy with multiple mucosal biopsies at the end of a double-blind placebo-controlled (DBPC) wheat challenge when they reported the reappearance of the symptoms. As controls we included 11 "healthy patients", sex- and age-matched with the patients who underwent colonoscopy evaluation for rectal bleeding due to hemorrhoids. Cells freshly obtained from rectal tissue were stained to detect anti-CD45, anti-CD3, anti-CD4, and anti-CD8. Furthermore, intracellular staining was performed with anti-tumor necrosis factor (TNF)-α, anti-interleukin (IL)-17, and anti-IL-22. Production of TNF-α by $CD45^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells, as well as of IL-17 by $CD4^+$ cells, was higher in the rectal tissue of NCWS patients than in controls. On the contrary, IL-22 production by $CD8^+$ cells was lower in NCWS patients than in the controls. In NCWS patients diagnosed by DBPC wheat challenge, there is a complex immunological activation, with a significant role for the adaptive response.

NEW & NOTEWORTHY Nonceliac wheat sensitivity (NCWS) is a syndrome characterized by symptoms triggered by gluten intake. The pathogenesis is still uncertain. Studies have shown a role for innate immunity. We demonstrated that production of TNF-α by $CD45^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells and of IL-17 by $CD4^+$ cells is higher in the rectal tissue of NCWS patients than in controls. We clearly demonstrated that in patients with NCWS there is a significant role for the adaptive response.

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adaptive immunity; IL-17; IL-22; nonceliac wheat sensitivity; TNF-α

INTRODUCTION

In recent years, a new gluten- or wheat-related disease has emerged, a condition labeled "nonceliac gluten sensitivity" (NCGS) or "nonceliac wheat sensitivity" (NCWS), as it is still not certain which of the components of the wheat is the real culprit in this pathology (11, 27). According to the Salerno Experts' Criteria, NCGS/NCWS is defined as a syndrome characterized by both intestinal and extraintestinal symptoms triggered by gluten intake, diagnosed when both celiac disease (CD) and IgE-mediated wheat allergy (WA) have been excluded, which resolves once gluten is eliminated from the diet (12).

NCWS pathogenesis is still uncertain, and it has been attributed to very different mechanisms: incomplete digestion and/or absorption of fermentable oligo- and disaccharides, monosaccharides and polyols (FODMAPs) (2), an inflammatory process driven by amylase-trypsin inhibitors (ATIs) (30), a psychological effect (25), and non-IgE-mediated food allergy (9), as well as other pathogenetic mechanisms (23, 34). The studies that showed a role for the immune system in NCWS pathogenesis have pointed to a predominant involvement of innate immunity (28, 29, 31), in keeping with animal and in vitro studies on ATI stimulation of the myelomonocytic compartment (36). Few studies, on the contrary, have investigated the activation of adaptative immunity and the role of lymphocytes in NCWS pathogenesis (3, 33). In a recent study, we demonstrated that in patients with active NCWS, type-1 innate lymphoid cells (ILC1s) infiltrate the rectal mucosa, express transcripts encoding the proinflammatory cytokine interferon (IFN)-γ, and decrease on a gluten (wheat)-free diet, supporting a role for this innate lymphoid cell subset in the pathogenesis of NCWS (14).

In the present pilot study, we aimed to study the different T-lymphocyte subsets in the rectal mucosa of NCWS symp-

tomatic patients to demonstrate the possible contribution of adaptative immune response in NCWS pathogenesis.

METHODS

Patients. We prospectively recruited adult patients with an irritable bowel syndrome (IBS)-like clinical presentation, according to the Rome III criteria, and a suspected diagnosis of NCWS. Most of the patients had been referred due to intestinal symptoms whose onset, they reported, was related to wheat ingestion, and most also had extraintestinal symptoms. The patients were recruited between January 2018 and January 2019 at two centers: Department of Internal Medicine at the University Hospital of Palermo, Italy, and Department of Internal Medicine of the Hospital of Sciacca, Agrigento, Italy.

During this period ~200 patients with suspected NCWS, very often with self-reported symptoms on wheat ingestion, were examined. After the exclusion of CD, WA, and inflammatory bowel disease (IBD) diagnoses, or other "organic" causes of symptoms, 58 received a definitive diagnosis of NCWS and consented to enter one or more of our ongoing study protocols. In these patients, NCWS diagnosis was based on the disappearance of symptoms on an elimination diet with the exclusion of wheat, and their reappearance on a subsequent double-blind placebo-controlled (DBPC) wheat challenge, using 80 g of wheat flour or rice flour for 2 wk (for details, see Supplemental File S1 at https://doi.org/10.6084/m9.figshare.12074181). Among the 58 subjects who received a definitive diagnosis of NCWS, 12 patients (11 women, 1 man, age range: 23–61 yr, median: 32 yr) were recruited at random for the present study and accepted to participate.

These patients underwent rectal endoscopy, with five mucosal biopsies taken at 5–15 cm from the anal verge, at the end of the DBPC challenge, when they reported the reappearance of symptoms.

Exclusion criteria were *1*) self-exclusion of wheat from the diet and refusal to reintroduce it before entering the study; and *2*) other "organic" gastrointestinal diseases.

As controls we included 11 "healthy patients" (10 women, 1 man, age range: 23–60 yr, median: 34 yr) who underwent colonoscopy evaluation for rectal bleeding. In all these subjects, colonoscopy, performed at the last ileal loop, was normal and rectal bleeding was due to hemorrhoids; they were consecutively recruited during the study period, being sex- and age-matched with the NCWS patients. All the control subjects had been regularly consuming a wheat-containing diet (with a daily amount of pasta plus bread ranging between 150 and 400 g) for at least 3 mo.

NCWS diagnosis. To diagnose NCWS, previously described criteria were adopted (6, 10) (see Supplemental File S1). Further inclusion criteria were *1*) age >18 yr; *2*) follow-up duration longer than 6 mo after the initial diagnosis; and *3*) at least two outpatient visits during the follow-up period.

Rectal biopsies enzymatic digestion and FACS analysis of mucosa infiltrating lymphocytes. Rectal biopsies were first transferred in RPMI 1640 medium (Euroclone, Devon, UK) with the addition of 20 mM HEPES (Euroclone), 100 U/mL penicillin (Euroclone), and 100 μg/mL streptomycin (Euroclone) to block bacterial growth and then minced into small pieces, followed by enzymatic digestion with 1.5 μg/ml type IV collagenase (Life Technologies, CA), 20 μg/ml hyaluronidase (Sigma, Merck KGaA, Darmstadt, Germany), and 50 μg/ml DNAase (Sigma) for 1 h at 37°C.

After digestion, we obtained a minimum number of 1,500,000 cells, and the subsequent experiments were performed in all patients on this yield size (1,500,000 cells); however, we did not normalize to the total amount of tissue. The harvested cells were washed twice in fresh medium supplemented with 2 mM L-glutamine (Euroclone) and 10% fetal calf serum (FCS, Euroclone) to block enzymatic digestion and then counted with the viability dye Trypan blue (Euroclone) and stained immediately. For intracellular analysis of cytokine expression, the Golgi blocker monensin (10 mg/ml) (Sigma) was added. Cells freshly obtained from rectal tissue were stained with the following fluorochrome-conjugated monoclonal antibodies (mAbs) for the detection of surface molecules: anti-CD45 (2D1, BioLegend, San Diego, CA), anti-CD3 (OKT3, BioLegend, San Diego, CA), anti-CD4 (OKT4, BioLegend), anti-CD8 (SK1, BioLegend), or isotype-matched control mAbs (BioLegend) in FACS buffer (PBS containing 2% FCS and 2 mM EDTA) for 30 min at 4°C. The Zombie fixable viability dye (BioLegend) was added to gate only CD45 live cells. At the end of this incubation, cells were washed once in FACS buffer, incubated for 30 min in a fixation solution (eBioscience, San Diego, CA), and thereafter permeabilized by centrifuging them for three times in an appropriate buffer (eBioscience). Permeabilized cells were then incubated with anti-interleukin (IL)-17 (eBio64CAP17, BioLegend), anti-tumor necrosis factor (TNF)-α (MAB1, BioLegend), anti-IL-22 (2G12A41, BioLegend), and isotype-matched control mAbs (BioLegend). Samples were acquired on a FACSCanto II (BD Biosciences, San Diego, CA) and analyzed using the FlowJo software (BD Biosciences).

Rectal histology study. Histopathological analysis was performed on formalin-fixed, paraffin-embedded rectal biopsy specimens. For rectal specimens, 4-μm-thick sections were routinely stained with hematoxylin and eosin (H&E) to assess the degree of inflammatory infiltration of the lamina propria, presence and number of lymphoid nodular aggregates, and eosinophil density.

The following parameters were evaluated: presence, number and size of lymphoid nodules and number of intraepithelial $CD3^+$ lymphocytes. Furthermore, in the lamina propria we counted the number of $CD45^+$ immunocytes; $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes; and eosinophils.

Immunohistochemical staining was performed with the BenchMark XT automated slide staining system (Ventana Medical Systems, Tucson, AZ), according to the manufacturer's instructions, using the following primary antibodies: CD3 (rabbit monoclonal, clone: 2GV6), CD4 (rabbit monoclonal, clone: SP35), CD8 (rabbit monoclonal, clone: SP57), and CD45-LCA (mouse monoclonal, clone: RP2/18). Negative controls without primary antibodies were included in each immunohistochemical run. The slides were analyzed under a Leica-DM2000 optical microscope (Leica Microsystems) using Leica ×4 SL, ×10 SL, HI PLAN ×20/0.40, HI PLAN ×40/0.65, HI PLAN ×63/0.75, and PL FLUOTAR ×100/1.30 objectives. Microphotographs were obtained using a Leica MC120 HD camera (Leica Microsystems).

Biopsy specimens were assessed by two of the authors (G. Geraci and/or A. M. Florena). All reviewers were blinded to the final diagnosis of each patient.

Ethics. This study was approved by the Ethics Committee of the University Hospital, Palermo, where the patients were recruited, and all of them gave written informed consent for the use and storage of tissue biopsies according to the Declaration of Helsinki principles. The study was registered at clinicaltrials.gov (registration no.: NCT01762579).

Statistical analysis. Continuous variables were described as median and range (minimum-maximum) and analyzed with the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant. All data were analyzed using GraphPad Prism version 6.0e (GraphPad, San Diego, CA).

RESULTS

Table 1 shows the individual clinical characteristics of the patients included in the study. Eight of the 12 NCWS patients completed the 2-wk wheat challenge period, although their symptoms significantly worsened during the challenge period (data not shown); they underwent rectal biopsies within 24 h after the end of the challenge. The other four NCWS patients did not complete the 2 wk of the challenge (stopping between 5 and 8 days), as they developed severe symptoms (abdominal

Table 1. *Individual clinical characteristics of 12 patients with NCWS included in the study*

Case	IBS-Type	Extraintestinal Symptoms	HLA DQ2/DQ8 Yes/No	Duodenal Histology	Colon Eosinophil Infiltration	AGA IgG/IgA	CD Family History	Associated Diseases	Other food Intolerances
1 AM	D	Anal fissure bleeding	DQ2	Marsh 1	YES	IgG positive	NO	Allergic rhinitis, iron deficiency anemia, systemic nickel allergy syndrome	Cow's milk, yeast
2 AA	D	Thoracic pain	DQ2	Marsh 0	YES	Positive	NO	Familial Mediterranean fever, autoimmune thyroiditis, iron deficiency anemia, gastroesophageal reflux disease, uterine fibromatosis	None
3 AM	A	Insomnia, asthenia	DQ2	Marsh 0	NO	Positive	NO	Dysmenorrhea	None
4 AA	D	Erythematous-crusty rash, myalgia, limb paresthesia, recurrent cystitis and vaginitis, hypoglycemia	DQ8	Marsh 0	YES	Negative	NO	Allergic asthma, Raynaud's phenomenon	Cow's milk
5 BF	D	NO	DQ2	Marsh 0	NO	Negative	NO	NO	Cow's milk, mushrooms and peppers
6 BMC	D	Headache, itching	DQ2	Marsh 1	NO	Negative	NO	Allergic rhinitis, uterine fibromatosis, lactose intolerance, systemic nickel allergy syndrome	Cow's milk
7 CMC	D	None	DQ2	Marsh 1	YES	Positive	NO	Autoimmune thyroiditis, arterial hypertension, hypercholesterolemia, osteoporosis, colon diverticulosis, contact dermatitis	Cow's milk
8 DM	A	Chronic fatigue, Brain fog	NO	Marsh 1	YES	Negative	NO	Asthma and allergic rhinitis	Yeast
9 DSPM	A	Face swelling	DQ2	Marsh 1	Not performed	Negative	NO	Rhinitis and allergic asthma, iron deficiency anemia, hypercholesterolemia	Potatoes, onion, peppers, lettuce, pear
10 BM	C	Headache, tiredness, Brain fog	NO	Marsh 0	Not performed	Negative	NO	Allergic rhinitis	None
11 SM	D	Headache	NO	Marsh 1	NO	IgG positive	NO	Iron deficiency anemia	Cow's milk
12 FB	C	NO	NO	Marsh 0	Not performed	Negative	NO	Allergic rhinitis	None

IBS, irritable bowel syndrome; IBS-D, diarrhea-predominant IBS; IBS-C, constipation-predominant IBS; IBS-A, IBS with alternating bowel habits; AGA, anti-gliadin antibodies; CD, celiac disease; NCWS, nonceliac wheat sensitivity; HLA, human leukocyte antigen.

pain, diarrhea, headache, and skin rash) starting on the first or second day of wheat consumption; they also underwent rectal biopsies within 24 h after ending their wheat challenge.

The cytofluorimetric study of the rectal biopsies from NCWS patients on wheat challenge showed a $CD45^+$ leukocyte infiltrate which was higher than in controls but not statistically significant (median: 18.2, range: 2.4–39.4% vs. median: 8.8, range: 2.1–24.5%, $P = 0.28$). Similarly, we did not find any statistically significant differences in $CD45^+$/$CD3^+$ and $CD45^+$/$CD3^-$ leukocyte infiltrate or in $CD3^+$/$CD4^+$ and $CD3^+$/$CD8^+$ leukocyte infiltrate between NCWS patients on wheat challenge and controls.

The histology study confirmed a trend for a higher $CD45^+$ and $CD45^+$/$CD3^+$ cell infiltrate in NCWS patients than in controls, although the cell count was not statistically significant. Figures 1 and 2 show a representative picture of the rectal mucosa of one of the NCWS patients and one of the controls in the study. In general, the rectal mucosa of the NCWS patients showed the presence of multiple intramucosal lymphoid nodules in several fragments, often with a secondary lymphoid follicle with evidence of a germinal center, located in the lamina propria of the mucosa. Lamina propria moderate chronic inflammation with an increase in eosinophilic granulocytes was also recorded in NCWS. However, none of the above differences between NCWS patients and control subjects were statistically significant.

Analysis of cytokine production demonstrated spontaneous TNF-α production by the $CD45^+$ cells of NCWS patients on wheat challenge, which was significantly higher than that of the controls (median: 1.71, range: 0.25–13.2% vs. median: 0.72, range: 0.34–1.53%, respectively, $P = 0.008$), whereas spontaneous IL-17 and IL-22 production by the $CD45^+$ cells of NCWS patients was comparable with that of the controls (Fig. 3*A*).

As shown in Fig. 3*B*, spontaneous TNF-α production was particularly marked in the $CD3^+$ cells of NCWS patients on wheat challenge compared with control subjects (median: 1.98, range: 0.57–13.80% vs. 0.83, range: 0.32–1.63%, $P = 0.004$), whereas spontaneous IL-17 and IL-22 production by $CD3^+$ cells of NCWS patients was comparable to that of controls. A representative FACS plot is shown in Supplemental File S2 (https://doi.org/10.6084/m9.figshare.12471503.v1).

Figure 4*A* shows the spontaneous TNF-α and IL-17 production by the $CD4^+$ cells of NCWS patients on wheat challenge in comparison with controls; $CD4^+$ of NCWS patients showed higher levels of TNF-α than controls (median: 0.43, range: 0.1–4.4% vs. median: 0.15, range: 0.0–0.63%, $P = 0.04$), and production of IL-17 was also higher (median: 0.48, range: 0.0–7.60% vs. median: 0.36, range: 0.0–0.58%, $P = 0.05$). On the contrary, spontaneous IL-22 production by $CD4^+$ cells was similar to controls (mean 0.48, range: 0.0–3.21% vs. 0.41, range: 0.0–4.58%, respectively).

Figure 4*B* shows cytokine production by $CD8^+$ cells. Spontaneous TNF-α production was higher in the $CD8^+$ cells of NCWS patients on wheat challenge than in controls (median: 0.83, range: 0.0–6.0% vs. 0.26, range: 0.0–1.0%, $P = 0.029$,). In contrast, IL-22 production was significantly lower in $CD8^+$

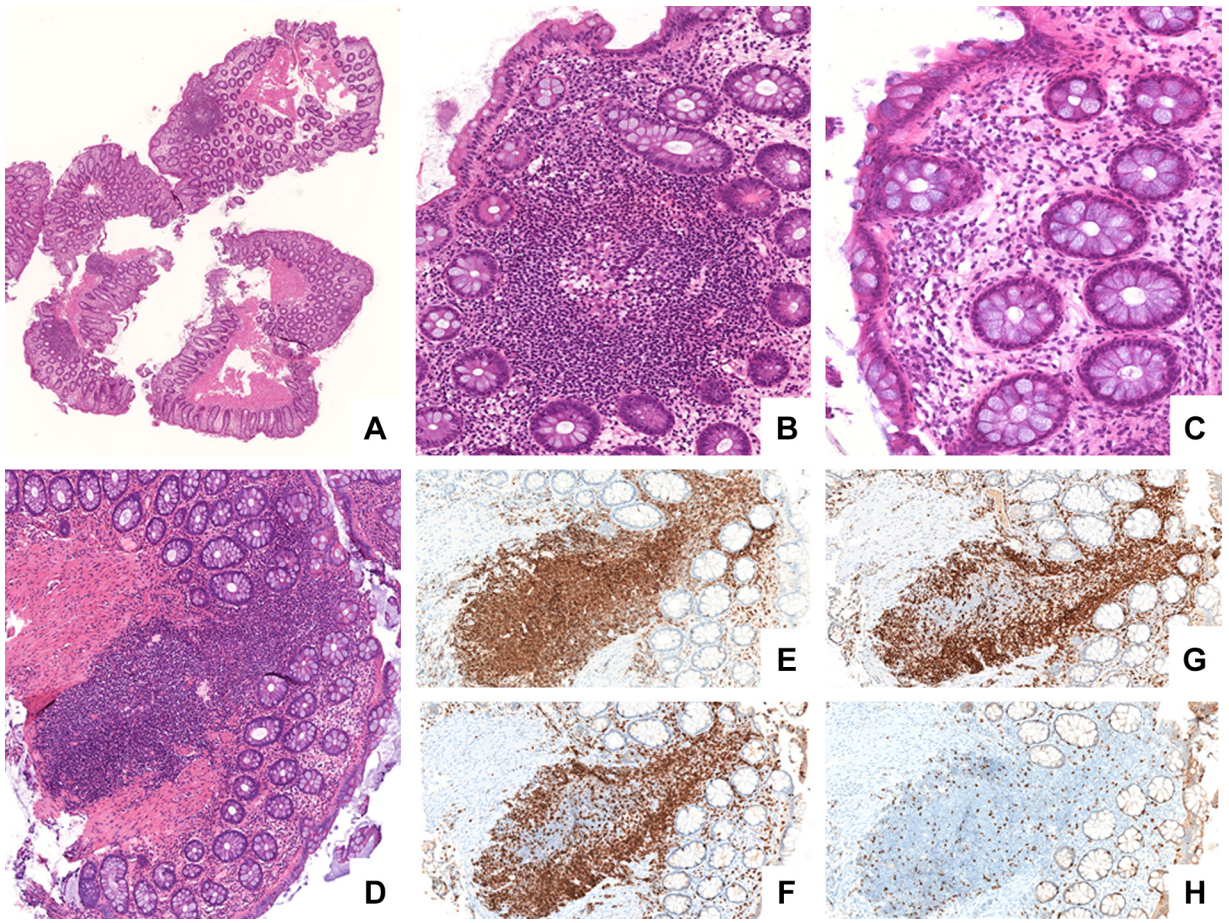

Fig. 1. Biopsies of rectal mucosa from 1 of the "nonceliac wheat sensitivity" (NCWS) patients included in the study. *A*: presence of multiple intramucosal lymphoid nodules in several fragments, readily evident at scan view. *B*: secondary lymphoid follicle, with a diameter of 528 μm, with evidence of a germinal center, located in the lamina propria of the mucosa (detail of micrograph *A*). *C*: lamina propria characterized by edema and moderate chronic inflammation, with moderate increase in eosinophilic granulocytes. *D*: in some NCWS patients, the nodular lymphoid infiltrate extended to the whole thickness of the mucosa. *E*: immunohistochemical stain for CD45. *F*: immunohistochemical stain for CD3. *G*: immunohistochemical stain for CD4. *H*: immunohistochemical stain for CD8. *A–D*: hematoxylin and eosin stain. Original magnification: *A*: ×10; *B* and *C*: ×100; *D*: ×150; *E–H*: ×100.

cells of NCWS patients than in controls (median: 0.18, range: 0.0–2.82% vs. median: 0.52, range: 0.0–3.40%, $P = 0.039$). Spontaneous IL-17 production by $CD8^+$ cells of NCWS patients on wheat challenge was comparable to controls (mean 0.37, range: 0.0–6.83% vs. median: 0.42, range: 0.0–2.69%, P = NS). For the representative FACS plots see Supplemental File S3 (https://doi.org/10.6084/m9.figshare.12471509.v1).

DISCUSSION

NCWS is emerging as a new clinical entity, although it is not yet well defined. The pathogenesis of the disease is likely to be the result of a complex interplay between different factors, including specific components of wheat and related cereals, intestinal barrier function, gut microbiota, and immunity (4, 5, 15, 20, 21). Increased intestinal permeability due to epithelial barrier damage, in particular, has been demonstrated in NCWS patients (28), and increased zonulin levels have also been correlated with abdominal pain, distension and anxiety (1). Recently, we contributed data supporting an immunologic effect of wheat ingestion, showing that NCWS patients have a strong tendency to autoimmunity, characterized by both associated autoimmune diseases and the presence of serum ANA positivity (6). Innate immunity (3, 28–31, 33, 36) has been suggested as being involved in the immunologic response of NCWS patients. Furthermore, experimental data have shown that components of wheat, ATIs, act as potent stimulators of innate immune reactions, via the stimulation of Toll-like receptor 4 (TLR4) in monocytes, macrophages, and dendritic cells (19). In this context, our previous data strongly support the role of innate immunity in the pathogenesis of NCWS, as they demonstrated a significant infiltration of $CD3^-$ cells in the rectal mucosa of NCWS patients and a dominant spontaneous IFN-γ production by these cells, which were further identified as an ILC1 population, expressing T-bet and producing IFN-γ (14).

In the present study, we did not find statistically significant differences in $CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$, and $CD3^-$ cell infiltration between NCWS patients and control subjects, even if $CD45^+$ cells were slightly higher in the rectal mucosa of NCWS than that of controls. This finding was observed both in the cytofluorimetric and the histology study. However, histology confirmed a trend towards an increase in eosinophil infiltration and the presence of large lymphoid nodules in the rectal mucosa of the NCWS patients, as previously reported (8),

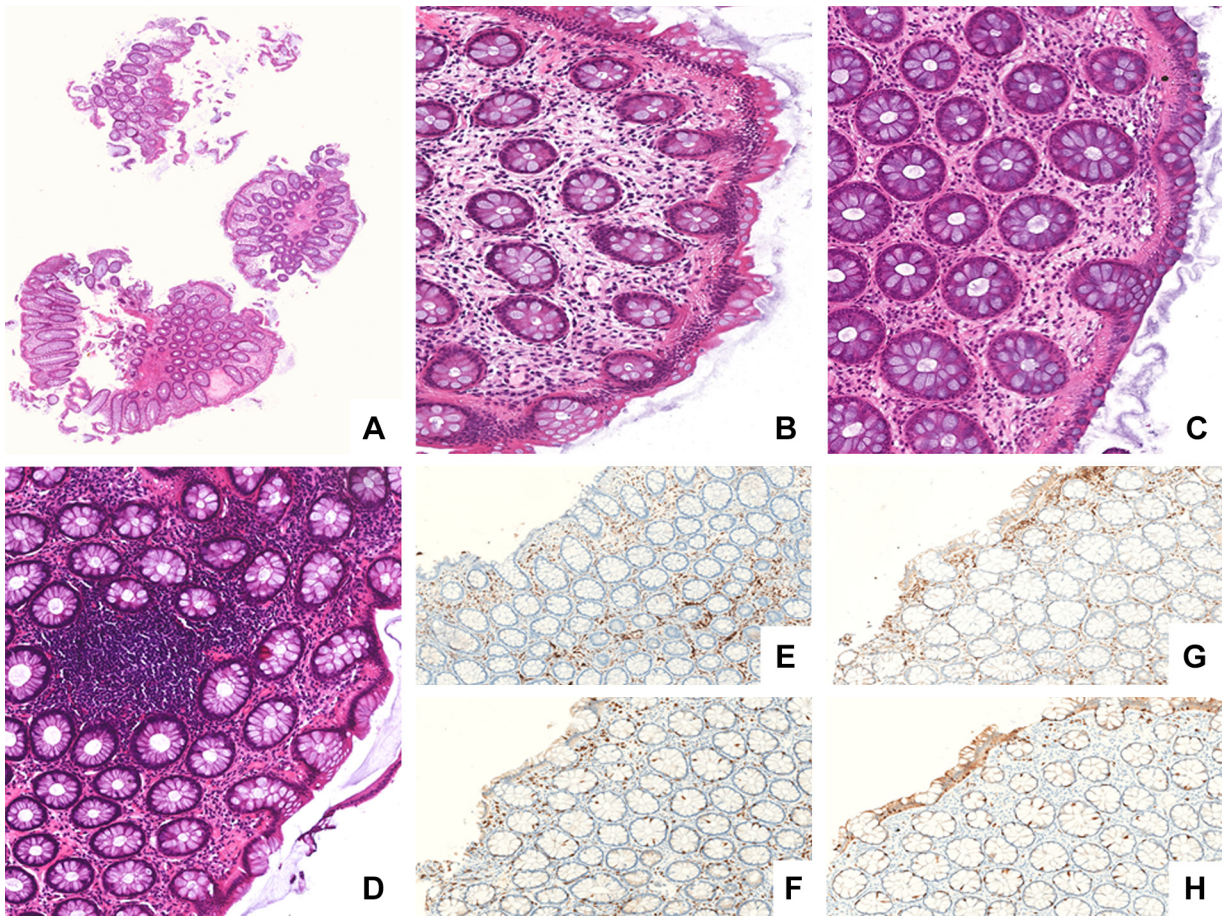

Fig. 2. Biopsies of rectal mucosa from one of the healthy controls included in the study. *A*: biopsy fragments more frequently showed substantially conserved architectural features. *B* and *C*: at higher magnification the lamina propria showed a mild inflammatory infiltrate, generally less intense than in the "nonceliac wheat sensitivity" (NCWS) patients. *D*: less frequently, lymphoid nodules were found in control subjects, which were less numerous and smaller in size than NCWS patients; the nodule shown in the photomicrograph has a diameter of 247 μm. *E*: immunohistochemical stain for CD45. *F*: immunohistochemical stain for CD3. *G*: immunohistochemical stain for CD4. *H*: immunohistochemical stain for CD8. *A–D*: hematoxylin and eosin stain. Original magnification: *A*: ×10; *B–D*: ×100; *E–H*: ×100.

although the difference was not statistically significant. In this respect, we suppose that the low number of study patients could have determined a beta error.

However, as regards cytokine production, we demonstrated here that production of TNF-α by $CD45^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells was higher in the rectal tissue of NCWS patients than of the control subjects. Similarly, production of IL-17 by $CD4^+$ cells in NCWS patients was higher than in the controls. On the contrary, IL-22 production by $CD8^+$ cells was lower in NCWS patients than in controls. These cytokine production modifications might contribute to the pathogenesis of the disease.

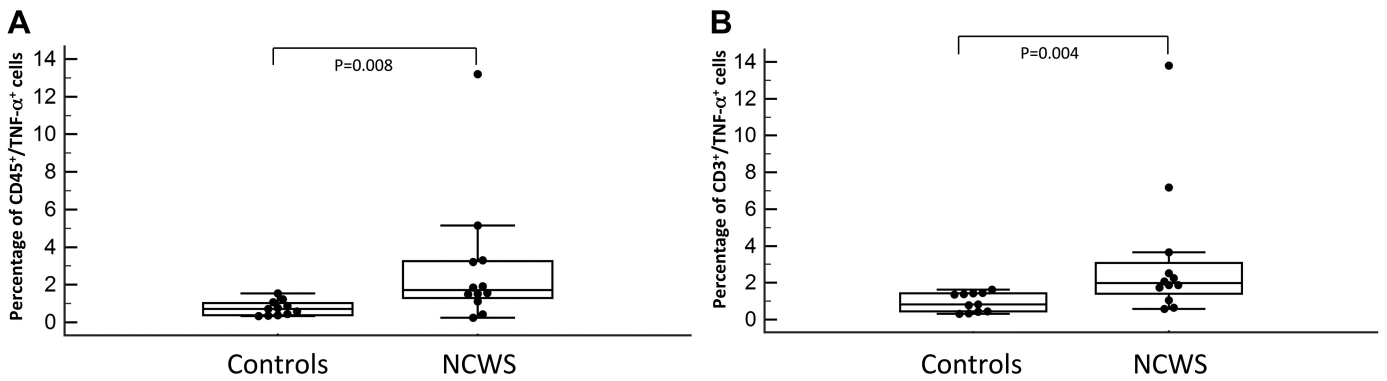

Fig. 3. *A*: TNF-α production by $CD45^+$ cells infiltrating the rectal mucosa of 12 "nonceliac wheat sensitivity" (NCWS) patients on wheat challenge and 11 healthy controls. *B*: TNF-α production by $CD3^+$ cells infiltrating the rectal mucosa of 12 NCWS patients on wheat challenge and 11 healthy controls. Data are expressed as median and interquartile range and points indicate the individual values. $P < 0.05$ (Mann-Whitney *U* test).

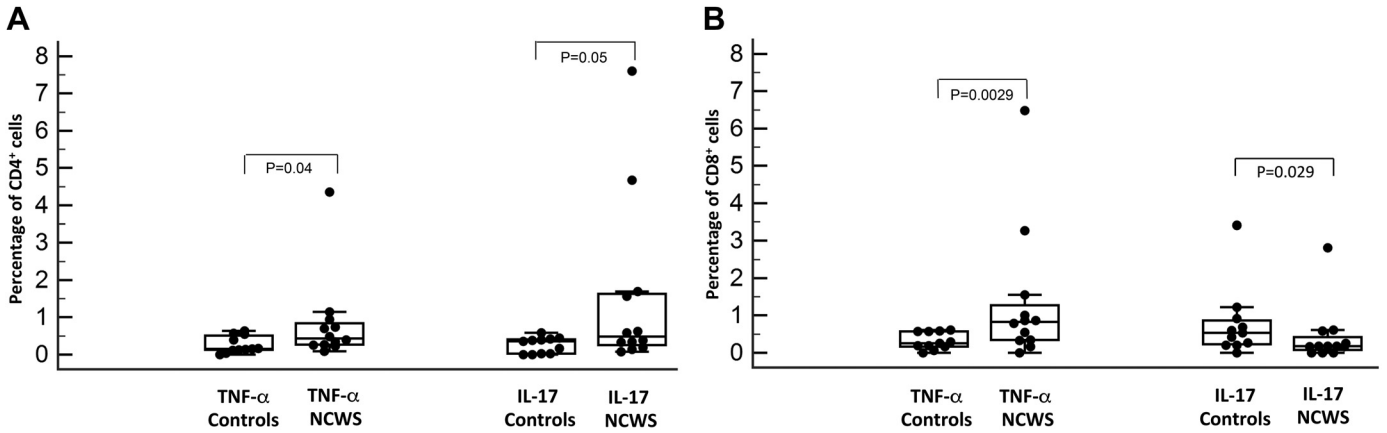

Fig. 4. *A*: TNF-α and IL-17 production by $CD4^+$ lymphocytes infiltrating the rectal mucosa of 12 "nonceliac wheat sensitivity" (NCWS) patients on wheat challenge and 11 healthy controls. *B*: TNF-α and IL-22 production of $CD8^+$ lymphocytes infiltrating the rectal mucosa of 12 NCWS patients on wheat challenge and 11 healthy controls. Data are expressed as median and interquartile range and points indicate the individual values. $P < 0.05$ (Mann-Whitney *U* test).

To our knowledge, this is the first report of an involvement of TNF-α in the pathogenesis of NCWS. TNF-α expression has been discovered in the colonic tissue and macrophages in both CD and IBD patients, and serum levels of TNF-α correlate with clinical and laboratory indices of intestinal disease activity (26). More recently, also IBS patients have been found to show higher TNF-α mucosal levels than controls, indicating that TNF-α could participate in IBS pathogenesis (32). In addition, analysis of IBS subgroups, based on symptomatology, has revealed subtle differences regarding TNF-α, showing a decrease in constipation-predominant IBS patients and an increase in diarrhea-predominant IBS patients (24). It is well known that NCWS patients present with IBS-like symptoms (22), and it could be hypothesized that $CD3^+$/TNF-α^+ cells may be characteristic of a subtype of IBS patients who really are suffering from NCWS or multiple food sensitivity, as recently shown by confocal endomicroscopy studies (7, 16, 17,). In any case, our data indicate that TNF-α might be involved in the pathogenesis of NCWS.

Furthermore, the data suggested that other cytokines, in particular IL-17 and IL-22, produced by $CD4^+$ and $CD8^+$ lymphocytes, may play a role in rectal mucosa inflammation in NCWS patients.

Production of IL-17 in our NCWS patient cohort might be related to TH17 lymphocyte activation. IL-17 is a cytokine related to the mechanisms of allergic reaction via its participation in the proliferation, maturation, and chemotaxis of neutrophils. In this context, Żbikowska-Gotz et al. demonstrated higher serum IL-17A concentrations in 30 patients with food allergy (diagnosed based on interview, clinical symptoms, positive skin prick test (SPT), placebo-controlled double-blind oral provocation trial, and the presence of IgE in blood serum against selected food allergens) than concentrations in 10 healthy volunteers (35). However, conflicting data exist and must be considered. Dhuban et al. (13), evaluating TH17 responses in 18 peanut-allergic children, who were also allergic to at least 1 additional food allergen, compared with 15 age-matched healthy controls, demonstrated a systemic, non-allergen-specific defect in TH17 response to antigen stimulation in food allergy individuals, suggesting a role for TH17 cells in the control of food allergy and implicating IL-17 as a potential biomarker for tolerance to food antigens. Our results also seem to point to an involvement of TH17 cells in NCWS pathogenesis, and this could be in keeping with our hypothesis that NCWS is an "allergic condition" (9) and also with a recent endomicroscopy study (16).

Several limits of our study must be underlined. First, we studied patients referred to tertiary centers with experience in CD and NCWS and this factor clearly led to a selection bias: therefore, our results cannot be extended to the broad population of self-diagnosed NCWS patients. Second, a very small patient sample was studied in this pilot, exploratory study: as stated above, a beta error could have occurred. Therefore, the results must be verified and confirmed with a larger number of cases, including patients suffering from IBS as controls. Third, our study did not look at the duodenal mucosa and we are not able to give data on this site. However, we chose to examine the colon and not the duodenal mucosa of our NCWS patients since they, as most of those included in our previous published studies, presented IBS-like symptoms. Consequently, we hypothesized that the immunologic response could be more prominent in the colon rather than in the duodenal mucosa (8, 10, 14). Fourth, we obviously did not consider many other potential players in the immune response in NCWS, i.e., mast cells have recently been demonstrated to be implicated in determining symptoms and nerve damage in NCWS (18). Neither did we study many other cytokine pathways, mainly those regarding a possible TH2 activation, which could also have a role in NCWS pathogenesis.

On the other hand, we clearly demonstrated that in patients suffering from NCWS, diagnosed by DBPC wheat challenge, there is a complex immunological activation, with a significant role for the adaptive response. It can be expected that "chronic" antigenic exposure might determine an initial innate response, and, in a second phase, an adaptive one, as the "first" and then "second" pathogenic phases of the disease, respectively. However, other research, with more patients and different kinds of controls, must be performed to better define the immunological pathogenesis of NCWS.

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We thank all the patients who agreed to participate in the study. The study was registered at Clinicaltrials.gov (registration no. NCT01762579), accessible at

https://www.clinicaltrials.gov/ct2/show/NCT01762579?cond=wheat+sensitivity&draw=3&rank=11.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.M., D.D.L., F.D., and A.C. conceived and designed research; D.D.L., F.F., G.G., A.G.G., A.S., A.D., F.L.B., M.L.P., A.M.F., and F.D. performed experiments; D.D.L. and M.S. analyzed data; P.M., D.D.L., F.F., F.D., and A.C. interpreted results of experiments; P.M., D.D.L., M.S., and A.G.G. prepared figures; P.M., D.D.L., F.D., and A.C. drafted manuscript; P.M. and A.C. edited and revised manuscript; P.M., D.D.L., F.D., and A.C. approved final version of manuscript.

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