Innate and adaptive immunity in self-reported nonceliac gluten sensitivity *versus* celiac disease

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ABSTRACT

Background: Immune mechanisms have been implicated in nonceliac gluten sensitivity (NCGS), a condition characterized by intestinal and/or extraintestinal symptoms caused by the ingestion of gluten in non-celiac/non-wheat allergic individuals.
Aims: We investigated innate and adaptive immunity in self-reported NCGS *versus* celiac disease (CD).
Methods: In the supernatants of *ex vivo*-cultured duodenal biopsies from 14 self-reported NCGS patients, 9 untreated and 10 treated CD patients, and 12 controls we detected innate cytokines – interleukin (IL)-15, tumor necrosis factor-α, IL-1β, IL-6, IL-12p70, IL-23, IL-27, IL-32α, thymic stromal lymphopoietin (TSLP), IFN-α-, adaptive cytokines – interferon (IFN)-γ, IL-17A, IL-4, IL-5, IL-10, IL-13-, chemokines – IL-8, CCL1, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL10-, granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF).
Results: Mucosal innate and adaptive cytokines, chemokines and growth factors did not differ between self-reported NCGS, treated CD and controls. On the contrary, IL-6, IL-15, IL-27, IFN-α, IFN-γ, IL-17A, IL-23, G-CSF, GM-CSF, IL-8, CCL1 and CCL4 were significantly higher in untreated CD than in self-reported NCGS, treated CD and controls, while TSLP was significantly lower in untreated CD than in self-reported NCGS, treated CD and controls.
Conclusion: In our hands, patients with self-reported NCGS showed no abnormalities of the mucosal immune response.

1. Introduction

Nonceliac gluten sensitivity (NCGS) is a clinical entity characterized by intestinal and/or extraintestinal symptoms due to the ingestion of gluten-containing foods in non-celiac/non-wheat allergic patients [1–3]. Unlike celiac disease (CD), which is induced in genetically susceptible individuals by both T helper cell type (Th)1/Th17-mediated adaptive[4,5] and innate immune mechanisms [6,7], NCGS has been presumed to be exclusively caused by abnormalities of the mucosal innate immune response (reviewed in Ref. [8]). The latter evidence was obtained in patients who did not undergo an oral double-blind, placebo-controlled gluten challenge test, and the concept of self-reported NCGS has been gaining international credibility [9,10]. Clinical features of patients who believe themselves to be gluten sensitive have been widely described in *ad hoc* studies [11,12].

On this basis, we aimed to perform a more in-depth investigation of a number of mucosal cytokines and chemokines, which are

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known to drive either the innate or the adaptive immune response in chronic intestinal inflammation, by detecting their levels in 24 h-cultured duodenal biopsy samples collected from patients with self-reported NCGS in comparison to those from CD patients.

2. Materials and methods

2.1. Patients and tissues

Well-oriented endoscopic biopsy specimens were collected from the second part of the duodenum of 14 subjects (mean age 37.5 years, range 20–62) referred to our Center because they were affected by self-reported NCGS, that is they complained of intestinal and/or extraintestinal symptoms which they themselves believed to be caused by gluten-containing food (Table 1). All 14 subjects had been under gluten-containing diet at the time of biopsy collection for at least two months. CD was ruled out on the basis of negative serum anti-endomysial and anti-tissue transglutaminase (tTG) antibodies and demonstration of normal duodenal histology, while they were under gluten-containing diet, whereas wheat allergy was excluded on the basis of negative serum specific IgE for wheat. Biopsies were also taken from nine consecutive patients with untreated CD (mean age 35.0 years, range 18–54), used as positive controls, and ten consecutive patients with CD after at least 12 months of gluten-free diet (GFD) (mean age 34.2 years, range 18–77), and from 12 control individuals (mean age 56.3 years, range 32–76) undergoing endoscopy for functional dyspepsia. All the control subjects did not complain of any intestinal and/or extraintestinal symptom related to the ingestion of gluten-containing foods, and they were all negative for anti-endomysial and anti-tTG antibodies and with normal histology. CD diagnosis was based on the positivity of serum anti-endomysial and anti-tTG antibodies associated with typical histopathological lesions, namely villous atrophy, increased intraepithelial lymphocyte (IEL) infiltration and crypt hyperplasia [13]. Among the nine untreated CD patients, seven showed a B2 lesion and two showed a B1 lesion [14]. Histological improvement was documented in all treated CD patients. After the perendoscopic collection of duodenal biopsies, 11 out of the 14 patients with self-reported NCGS underwent an oral double-blind, placebo-controlled, cross-over gluten challenge trial (for trial description, see Ref. [15]). Briefly, these patients belonged to a cohort of 61 patients randomly assigned to two groups given a 1-week treatment with 4.375 g/day of purified wheat gluten or rice starch (placebo), both administered *via* gastrosoluble capsules. Gluten capsules were free of fermentable, oligo-, di- and monosaccharides and polyols. After a 1-week wash-out period, participants crossed over to another week of placebo or gluten treatment, respectively. During the trial, patients filled in a daily questionnaire in order to evaluate a rating scale of both intestinal and extraintestinal symptoms. Hence, for each of these 11 patients with self-reported NCGS we calculated the overall (intestinal *plus* extraintestinal) score under gluten and the *delta* overall score, obtained by subtracting the weekly overall score under placebo from the weekly overall score under gluten. All the subjects included in the study were tested to rule out gastrointestinal infections, and they were not taking any kind of medication. Biopsies were processed for routine histology or organ culture. Each patient who took part in the study gave informed consent, and Ethics Committee approval was obtained in all cases.

2.2. Duodenal histology

Sections were taken from paraffin blocks of biopsies and stained with hematoxylin–eosin and with immunoperoxidase using anti-CD3 antibody (1:100 dilution, clone PS1, Novocastra, Newcastle,

Table 1
Clinical data of the 14 patients with self-reported nonceliac gluten sensitivity.

At baseline										Double-blind, placebo-controlled, cross-over trial		
Pt	Age	Sex	Duration of symptoms (months)	Previous self-prescribed GFD	Family history of CD	HLA-DQ2/DQ8	Serum IgG AGA	Anti-tTG2 IgA antibody deposits	IEL (%)	Overall score under gluten	Overall score under placebo	*Delta* overall score[a]
1	43	F	42	Yes	No	Negative	Negative	NA	24	15	20	−5
2	43	F	84	Yes	No	Negative	Negative	Absent	23	64	29	+35
3	47	F	14	Yes	No	Positive	Negative	Absent	34	56	85	−29
4	49	F	25	Yes	No	Negative	Positive	Absent	27	71	16	+55
5	42	F	15	Yes	No	Negative	Negative	Absent	45	107	65	+42
6	52	F	4	No	No	Positive	Negative	Absent	15	86	69	+17
7	20	F	38	Yes	No	Negative	Negative	Absent	38	NR	NR	NR
8	31	F	3	No	No	Positive	Negative	Absent	28	24	49	−25
9	37	F	12	No	No	Negative	Negative	Absent	30	18	8	+10
10	53	F	8	No	No	Negative	Negative	Absent	14	NR	NR	NR
11	21	F	33	No	No	Negative	Negative	Absent	21	58	154	−96
12	33	F	84	Yes	No	Negative	Negative	Absent	18	NR	NR	NR
13	22	F	11	Yes	Yes	Positive	Negative	NA	21	37	18	+19
14	62	M	8	No	No	Negative	Negative	NA	40	24	15	+9

[a] *Delta* overall score is calculated by subtracting the weekly overall score under placebo from the weekly overall score under gluten. AGA, anti-gliadin antibodies; CD, celiac disease; F, female; GFD, gluten-free diet; HLA, human leukocyte antigen; IEL, intraepithelial lymphocyte; M, male; NA, not available; NR, not randomized in the trial; Pt; patient; tTG, tissue transglutaminase.

UK). IEL number was counted as CD3-positive cells *per* 100 intestinal epithelial cells evaluating at least 500 intestinal epithelial cells. The number of eosinophils in the lamina propria in each high power field (HPF) was counted as the peak of eosinophil count (the highest number of eosinophils per HPF; 40× objective).

2.3. Direct double immunofluorescence

Mucosal anti-tTG2 IgA antibody deposits were investigated by direct double immunofluorescence according to a method described previously [16]. Briefly, six frozen duodenal sections per patient were incubated with a mouse anti-human tTG2 antibody (1:100 dilution; CUB7402, NeoMarkers, USA) followed by an Alexa fluor 594-conjugated donkey anti-mouse IgG secondary antibody (1:100 dilution; Eugene, USA) and then by a fluorescein rabbit anti-human IgA antibody (1:80 dilution; Dako, Denmark). The multicolor analysis was performed using an Axioplan2 fluorescence microscope (Carl Zeiss, Germany) to localize the anti-tTG2 IgA antibody deposits.

2.4. Organ culture

Biopsies were placed in 24-well plates (VWR International, Lutterworth, UK) in 300 μl serum-free HL-1 medium (Cambrex Bio-Science, Wokingham, UK) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, and cultured at 37 °C, 5%CO_2. After 24 h culture, biopsies and supernatants were snap frozen and stored at −70 °C.

2.5. Cytokine array

Using the Proteome Profiler® Array Panel A kit (R&D Systems, Abingdon, UK) organ culture supernatants were analyzed for their cytokine content, including tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-12p70, IL-23, IL-27, IL-32α, interferon (IFN)-γ, IL-17A, IL-4, IL-5, IL-10, IL-13, IL-8, CCL1, CCL2, CCL3, CCL4, CCL5 CXCL1, CXCL10, granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). Briefly, 150 μl of supernatants were diluted and mixed with a cocktail of biotinylated detection antibodies. Each sample/antibody mixture was then incubated onto a nitrocellulose membrane coated with specific capture antibodies. Any cytokine/detection antibody complex present was bound by its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, streptavidin-HRP and chemiluminescent detection reagents were added sequentially, and pixel density of duplicate spots were quantified by scanning densitometry and ImageJ software (National Institute of Mental Health, Bethesda, MD). Pixel density of each analyte for any sample was normalized for averaged positive controls of the nitrocellulose membrane and then expressed as ‰.

2.6. ELISA

IL-15 concentration was measured in organ culture supernatants using the IL-15 ELISA kit (R&D Systems), according to the manufacturers' instructions.

2.7. Immunoblotting

Immunoblotting was performed according to a modified method described previously [17]. Proteins (100 μg) from 24 h-cultured duodenal biopsies were loaded and subjected to 10% SDS-PAGE under reducing conditions, followed by nitrocellulose (Bio-Rad Laboratories, Hercules, California, USA) transfer. Mouse anti-human IFN-α (1:1000 dilution; Proteintech, Manchester, UK) was used as primary antibody. Horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000 dilution; DAKO, High Wycombe, UK) was then used as secondary antibody, and the reaction was developed with the ECL plus kit (Amersham Biosciences, Little Chalfont, UK). Blots were stripped and analyzed for internal loading control using rabbit anti-β-actin antibodies (1:5000 dilution; Abcam, Cambridge, UK). Bands were quantified using an LKB Ultrascan XL Laser Densitometer (Kodak, Hemel Hempstead, UK).

2.8. RNA extraction and analysis of mRNA expression by quantitative RT-PCR

Reverse transcription (Im-PromII, Promega, Southampton, UK) on 1 μg total RNA extracted from 24 h-cultured duodenal biopsies was performed, and cDNA was used for PCR. Primer sequences were as follows: thymic stromal lymphopoietin (TSLP) forward, 5′-CCCAGGCTATTCGGAAACTCAG-3′, and reverse, 5′-CGCCACAATCCTTGTAATTGTG-3′; IFN-γ forward, 5′-GTATTGCTTTGCGTTGGACA-3′, and reverse, 5′-GAGTGTGGAGACCATCAAGGA-3′. Typically, 40 cycles of 20 s at 95 °C and 20 s at 60 °C were followed by the thermal dissociation protocol for Fast SYBR green detection. PCR reactions were normalized by expression analysis of cytokeratin 18 (CK18) with the following primers: forward, 5′-TGATGACACCAATATCACACGAC-3′, and reverse, 5′-TACCTCCACGGTCAACCCA-3′ [18].

2.9. Statistical analysis

Data were analyzed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) using the non-parametric Mann–Whitney *U* test or the Spearman's correlation. A level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. IELs and lamina propria eosinophils

A few IELs were observed in self-reported NCGS (Fig. 1A), whereas marked duodenal lymphocytosis was evident in the atrophic mucosa of untreated CD (Fig. 1B). Similarly to self-reported NCGS, a few IELs were identified in the mucosa of treated CD patients (Fig. 1C) and controls (Fig. 1D). As shown in Fig. 1E, the percentage of IELs did not differ in self-reported NCGS patients (median 25.5%, range 14.0–45.0) compared to treated CD patients (median 22.0%, range 17.0–31.0) and control subjects (median 24.0%, range 16.0–42.0). The percentage of IELs was significantly ($p < 0.0005$) higher in the duodenum of untreated CD patients (median 45.0%, range 32.0–50.0) in comparison to self-reported NCGS patients, treated CD patients and control subjects. A few eosinophils scattered in the lamina propria were evident in the mucosa of all four groups (Fig. 1F–I). As shown in Fig. 1J, the number of eosinophils did not significantly differ between self-reported NCGS (median 18.0, range 5.0–45.0), untreated CD (median 20.0, range 15.0–60.0), treated CD (median 18.0, range 9.0–32.0) and control subjects (median 17.0, range 1.0–35.0). No significant correlation was found in self-reported NCGS patients undergoing double-blind, placebo-controlled, cross-over trial between IEL or eosinophil number and either overall score under gluten or *delta* overall score (Supplementary Table 1).

3.2. Anti-tTG IgA antibody deposits

No mucosal anti-tTG IgA antibody deposits were detected in any of the self-reported NCGS patients (Supplementary figure* 1A), whereas they were evident in the duodenal mucosa of untreated CD patients (Supplementary figure* 1B), and disappeared after GFD (Supplementary figure* 1C).

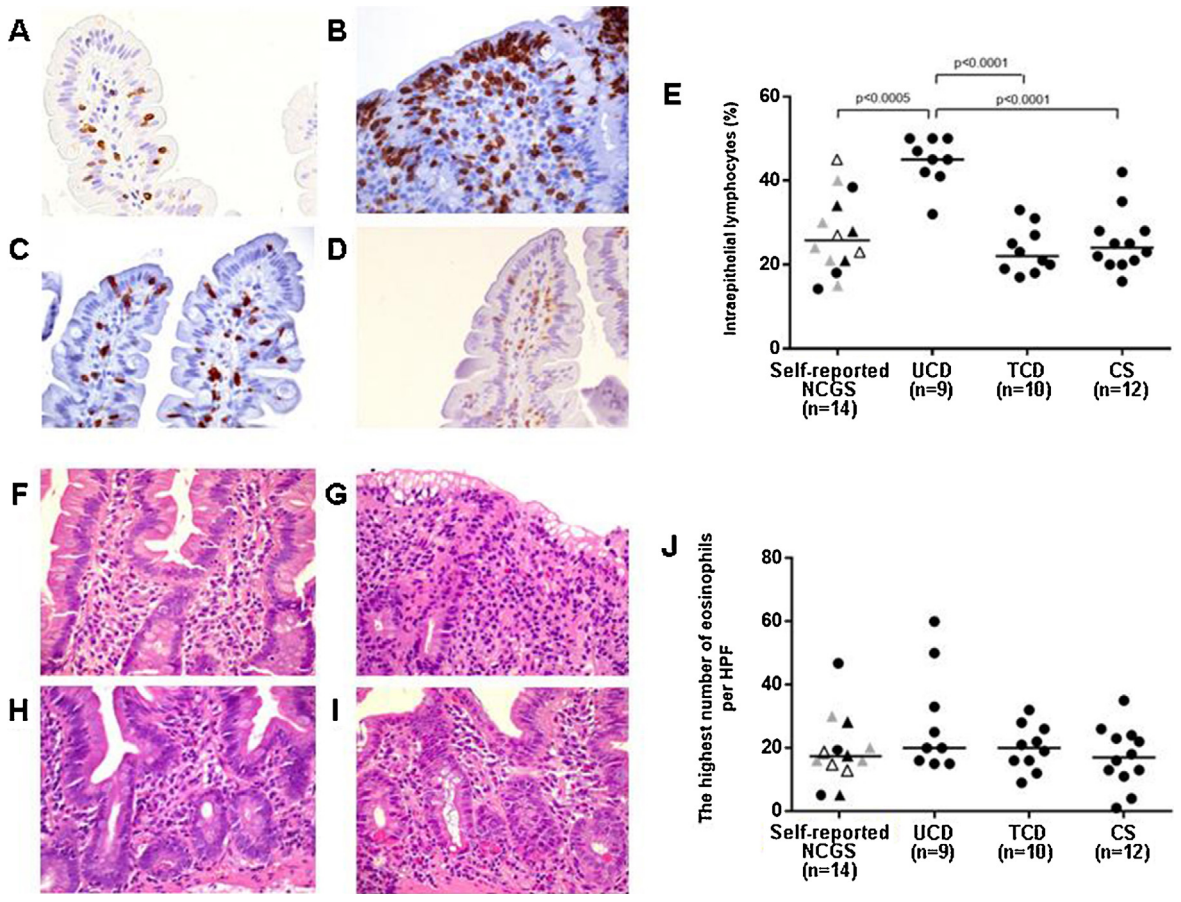

Fig. 1. Intraepithelial lymphocytes (IELs) and lamina propria eosinophils. In sections stained for CD3 (original magnification 40×), a few IELs were evident in a patient with self-reported nonceliac gluten sensitivity (NCGS) (A), whereas IEL infiltration was marked in a patient with untreated celiac disease (UCD) (B). CD3-positivity was limited to a few cells at intraepithelial layer both in a patient with treated celiac disease (TCD) (C) and in a control subject (CS) (D). Data are representative of staining performed in 14 self-reported NCGS, nine UCD, ten TCD patients and 12 CS. A few eosinophils scattered in the lamina propria were evident in a patient with self-reported NCGS (F), a patient with UCD (G), a patient with TCD (H) and a CS (I) (original magnification 40×). Data are representative of staining performed in 14 self-reported NCGS, nine UCD, ten TCD patients and 12 CS. In panel E (IELs) and J (eosinophils), patients undergoing double-blind, placebo-controlled, cross-over trial are indicated with white, gray or black triangles according to whether they had the highest (>+20), intermediate (between +20 and −20) or the lowest (<−20) *delta* overall score, calculated by subtracting the weekly overall score under placebo from the weekly overall score under gluten, respectively. Horizontal bars are median values.

3.3. Ex vivo *production of innate cytokines*

As shown in Fig. 2, production of TNF-α, IL-1β, IL-6, IL-12p70, IL-15, IL-23, IL-27 and IL-32α did not significantly differ in self-reported NCGS patients compared to treated CD patients and control subjects. The amount of IL-6, IL-23 and IL-27 was significantly higher in the supernatants of untreated CD biopsies in comparison to those of self-reported NCGS ($p < 0.05$), treated CD patients ($p < 0.05$) and control subjects ($p < 0.01$). A significantly ($p < 0.05$) higher concentration of IL-15 was found in untreated CD supernatants (mean 568 ± 196 pg/ml) in comparison to self-reported NCGS (mean 326 ± 166 pg/ml), treated CD (mean 292 ± 143 pg/ml) and control (mean 235 ± 155 pg/ml) supernatants. Furthermore, levels of TNF-α, IL-1β, IL-12p70, and IL-32α in untreated CD supernatants did not differ compared to self-reported NCGS, treated CD patients and control supernatants. As IFN-α is over-produced in CD mucosa [19], we determined its protein levels by immunoblotting in cultured duodenal biopsies of self-reported NCGS, untreated CD and treated CD patients, and control subjects (Supplementary figure* 2A). IFN-α protein did not differ between self-reported NCGS patients, treated CD patients and control subjects. As expected, a significantly ($p < 0.05$) higher IFN-α amount was observed in the mucosa of untreated CD compared with self-reported NCGS, treated CD and controls. As TSLP is known to be decreased in untreated CD [19], we then evaluated mucosal TSLP transcripts by qRT-PCR (Fig. 3). Upon normalization for CK18, no significant difference was found in TSLP transcripts between self-reported NCGS patients, treated CD patients and control subjects. As expected, TSLP mRNA was significantly reduced in untreated CD compared to self-reported NCGS ($p < 0.005$), treated CD ($p < 0.0001$) and control subjects ($p < 0.0005$). No significant correlation was found in self-reported NCGS patients undergoing double-blind, placebo-controlled cross-over trial between innate cytokine levels and either overall score under gluten or *delta* overall score (Supplementary Table 1).

3.4. Ex vivo *production of adaptive cytokines*

As shown in Fig. 4, production of IFN-γ, IL-17A, IL-4, IL-5, IL-10 and IL-13 did not significantly differ in self-reported NCGS patients compared to treated CD patients and control subjects. The Th1 cytokine IFN-γ and the Th17 cytokine IL-17A were significantly ($p < 0.05$) higher in untreated CD supernatants in comparison to self-reported NCGS, treated CD and control supernatants. As a previous study [10] showed an increase in IFN-γ transcripts in self-reported NCGS patients after a short-term challenge with gluten, we also assessed IFN-γ mRNA by qRT-PCR (Supplementary figure* 2B). Upon normalization for CK18, no significant difference was found in IFN-γ transcripts between self-reported NCGS patients, treated CD patients and control subjects. As expected,

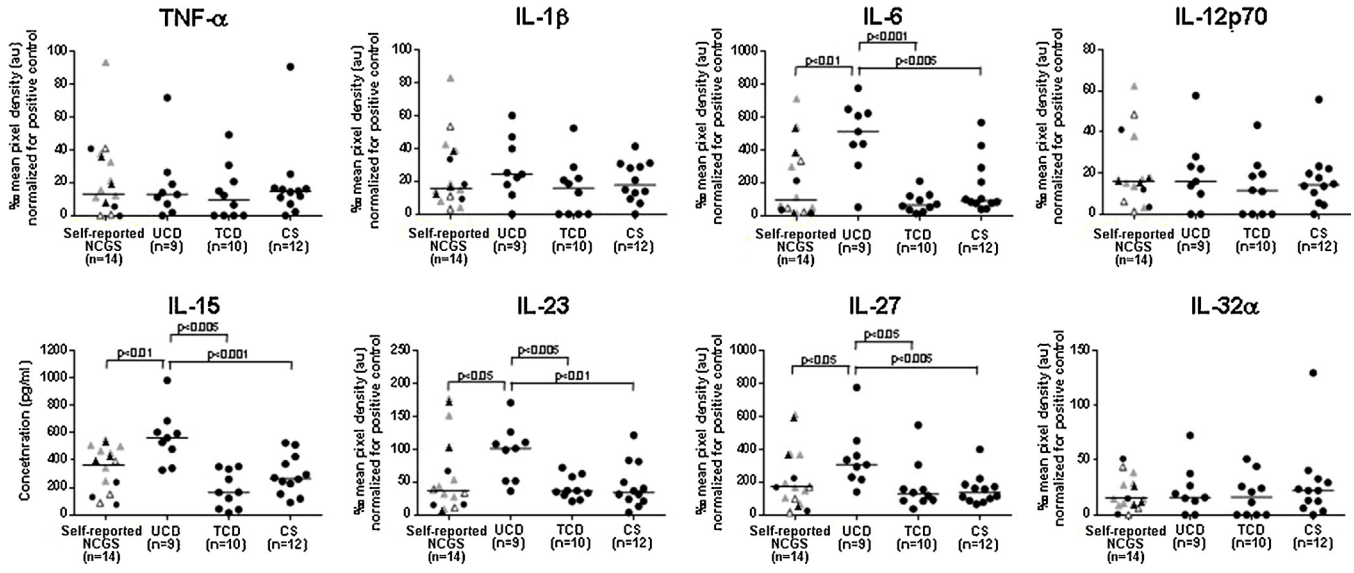

Fig. 2. *Ex vivo* production of innate cytokines. Levels of TNF-α, IL-1β, IL-6, IL-12p70, IL-23, IL-27 and IL-32α expressed as ‰ mean pixel intensity normalized for averaged positive controls of the nitrocellulose membrane, were measured by cytokine array in the biopsy supernatants of 14 self-reported nonceliac gluten sensitivity (NCGS) patients, nine untreated celiac disease (UCD) patients, ten treated celiac disease (TCD) patients and 12 control subjects (CS) and cultured for 24 h in the absence of *stimuli*. Levels of IL-15, expressed in pg/ml, were measured by ELISA. Patients undergoing double-blind, placebo-controlled, cross-over trial are indicated with white, gray or black triangles according to whether they had the highest (>+20), intermediate (between +20 and −20) or the lowest (<−20) *delta* overall score, calculated by subtracting the weekly overall score under placebo from the weekly overall score under gluten, respectively. Horizontal bars are median values.

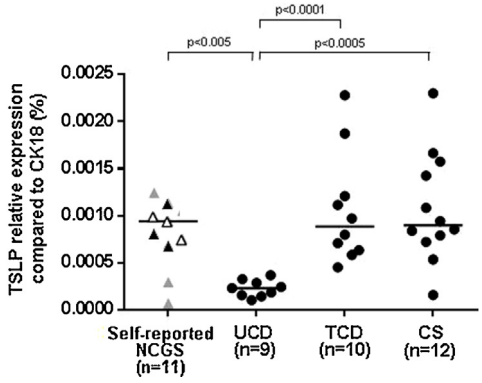

Fig. 3. *Ex vivo* production of TSLP. TSLP transcripts were quantified by qRT-PCR in the cultured duodenal biopsies of 11 patients with self-reported nonceliac gluten sensitivity (NCGS), nine patients with untreated celiac disease (UCD), ten patients with treated celiac disease (TCD) and 12 control subjects (CS). Changes in transcript levels were normalized for cytokeratin 18 (CK18). Patients undergoing double-blind, placebo-controlled, cross-over trial are indicated with white, gray or black triangles according to whether they had the highest (>+20), intermediate (between +20 and −20) or the lowest (<−20) *delta* overall score, calculated by subtracting the weekly overall score under placebo from the weekly overall score under gluten, respectively. Horizontal bars are median values. au, arbitrary units.

IFN-γ mRNA was significantly ($p < 0.0001$) increased in untreated CD compared to self-reported NCGS, treated CD and control subjects. Moreover, the amount of the Th2 cytokines IL-4, IL-5, IL-10 and IL-13 in untreated CD supernatants did not significantly differ compared to self-reported NCGS patients, treated CD patients and control supernatants. No significant correlation was found in NCGS patients undergoing double-blind, placebo-controlled, cross-over trial between adaptive cytokine levels and either overall score under gluten or *delta* overall score (Supplementary Table 1).

3.5. Ex vivo *production of chemokines*

As shown in Fig. 5, production of IL-8, CCL1, CCL2, CCL3, CCL4, CCL5, CXCL1 and CXCL10 did not significantly differ in self-reported NCGS patients in comparison to treated CD patients and control subjects. The amount of IL-8, CCL1 and CCL4 was significantly higher in untreated CD supernatants in comparison to NCGS ($p < 0.05$), treated CD ($p < 0.05$) and control supernatants ($p < 0.005$). Release of CCL2, CCL3, CCL5, CXCL-1 and CXCL10 in untreated CD supernatants did not significantly differ compared to self-reported NCGS patients, treated CD patients and control subjects. No significant correlation was found in NCGS patients undergoing double-blind, placebo-controlled, cross-over trial between chemokine levels and either overall score under gluten or *delta* overall score (Supplementary Table 1).

3.6. Ex vivo *production of growth factors*

G-CSF and GM-CSF release did not significantly differ in self-reported NCGS patients in comparison to treated CD patients and control subjects (Supplementary figure* 3). The amount of G-CSF and GM-CSF was significantly ($p < 0.05$) higher in untreated CD supernatants in comparison to self-reported NCGS, treated CD and control supernatants. No significant correlation was found in self-reported NCGS patients undergoing double-blind, placebo-controlled, cross-over trial between growth factor levels and either overall score under gluten or *delta* overall score (Supplementary Table 1).

4. Discussion

A considerable amount of conflicting evidence has recently been provided on the mechanisms presumed to play a role in the pathogenesis of NCGS, including intestinal permeability [21–23] and innate immunity [10,22,24,25]. Abnormalities of the innate immunity have been implicated on the basis of an increased IEL infiltration, up-regulated Toll-like receptor 2 and 4 transcript levels, and reduced transcript levels of the regulatory T cell marker Foxp3 in the duodenal mucosa of NCGS patients [22,24]. Conversely, gliadin does not induce *ex vivo* inflammation in organ culture biopsies or *in vitro* activation of peripheral basophils from NCGS patients [25], and *in vivo* short-term gluten challenge of NCGS

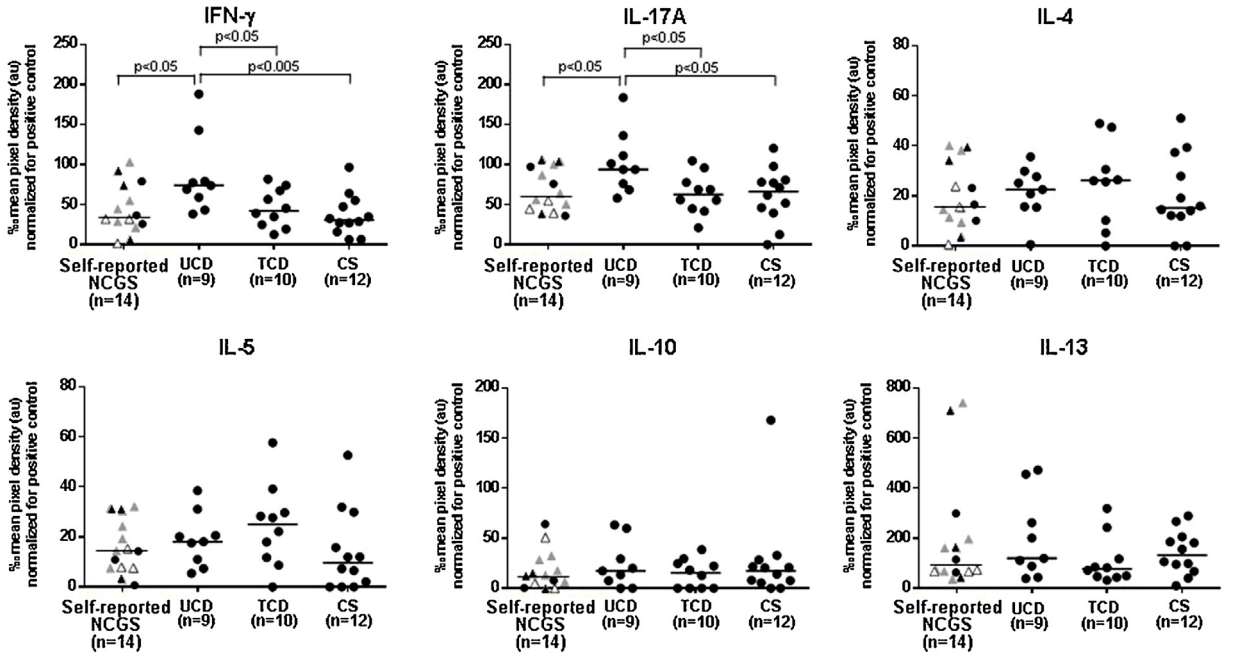

Fig. 4. *Ex vivo* production of adaptive cytokines. Levels of IFN-γ, IL-17A, IL-4, IL-5, IL-10 and IL-13, expressed as ‰ mean pixel intensity normalized for averaged positive controls of the nitrocellulose membrane, were measured by cytokine array in the biopsy supernatants of 14 self-reported nonceliac gluten sensitivity (NCGS) patients, nine untreated celiac disease (UCD) patients, ten treated celiac disease (TCD) patients and 12 control subjects (CS) and cultured for 24 h in the absence of *stimuli*. Patients undergoing double-blind, placebo-controlled, cross-over trial are indicated with white, gray or black triangles according to whether they had the highest (>+20), intermediate (between +20 and −20) or the lowest (<−20) *delta* overall score, calculated by subtracting the weekly overall score under placebo from the weekly overall score under gluten, respectively. Horizontal bars are median values.

mucosa does not change transcript levels of IL-8 and CCL2 [10], two chemokines implicated in recruiting immune cells in the inflamed gut [26].

On the basis of these discrepant findings, we aimed to verify whether innate and adaptive immune pathways are altered in the duodenal mucosa of patients with self-reported NCGS. When we detected the innate cytokines TNF-α, IL-1β, IL-6, IL-12p70, IL-15, IL-23, IL-27, and IL-32α, we found that none of them were abnormally produced by self-reported NCGS biopsies. While for IL-6 and TNF-α the results were expected on the basis of previous studies showing their normal mucosal concentration in NCGS patients [10,22], these are the first data demonstrating unchanged IL-1β, IL-12p70, IL-15, IL-23, IL-27 and IL-32α levels in this condition. As IFN-α is up-regulated and drives Th1 response in CD mucosa [19],

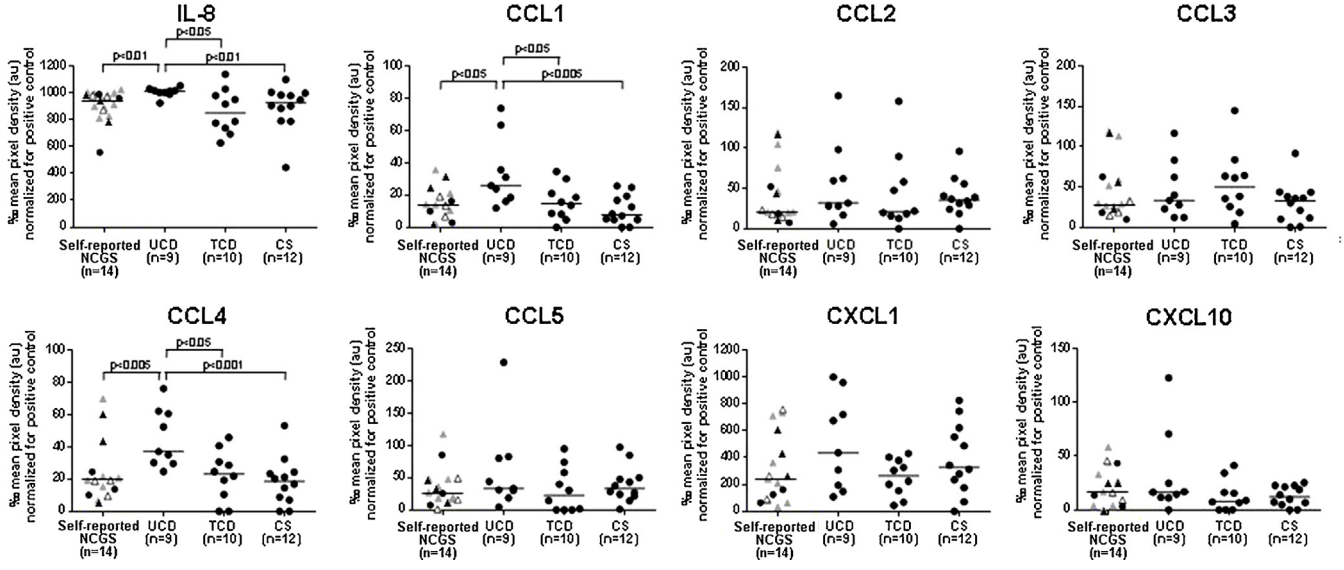

Fig. 5. *Ex vivo* production of chemokines. Levels of IL-8, CCL1, CCL2, CCL3, CCL4, CCL5, CXCL1 and CXCL10, expressed as ‰ mean pixel intensity normalized for averaged positive controls of the nitrocellulose membrane, were measured by cytokine array in the biopsy supernatants of 14 self-reported nonceliac gluten sensitivity (NCGS) patients, nine untreated celiac disease (UCD) patients, ten treated celiac disease (TCD) patients and 12 control subjects (CS) and cultured for 24 h in the absence of *stimuli*. Patients undergoing double-blind, placebo-controlled, cross-over trial are indicated with white, gray or black triangles according to whether they had the highest (>+20), intermediate (between +20 and −20) or the lowest (<−20) *delta* overall score, calculated by subtracting the weekly overall score under placebo from the weekly overall score under gluten, respectively. Horizontal bars are median values.

we detected this cytokine in self-reported NCGS mucosa where its levels were found to be normal. We also measured TSLP, an epithelial-derived immunoregulatory cytokine shown to be down-regulated in untreated CD [20], and its transcripts were found unchanged in self-reported NCGS. Of note, we did find a significantly higher release of IL-23 and IL-27 from untreated CD biopsies in comparison to treated CD and control biopsies. The increase in IL-23 is not surprising in the light of the well-known implication of IL-17A in active CD [5], whereas the over-production of IL-27 might be involved in sustaining the abnormal Th1 response underlying villous atrophy in untreated CD [4].

As regards adaptive immunity, in agreement with Sapone et al. [22,24] we showed no difference in IFN-γ and IL-17A in self-reported NCGS in comparison to controls, although both these cytokines were significantly up-regulated in untreated CD in comparison to treated CD and controls in keeping with previous studies [4,5]. No data are available in the literature concerning all the remaining adaptive cytokines, *i.e.* IL-4, IL-5, IL-10 and IL-13, which were found unchanged in the self-reported NCGS supernatants. We also demonstrated that the spontaneous production by self-reported NCGS biopsies of G-CSF and GM-CSF, two growth factors implicated in prolonging the survival of monocytes, macrophages and neutrophils [27], was comparable to that of control mucosa. Our results are in apparent discrepancy with those of Vazquez-Roque et al. [23] who showed increased GM-CSF levels in response to *in vitro* gluten stimulation, although these results were obtained from peripheral and not mucosal mononuclear cells, and from nonceliac diarrhea-predominant irritable bowel syndrome patients rather than NCGS patients. Conversely, both G-CSF and GM-CSF were significantly up-regulated in the supernatants of untreated CD biopsies in comparison to treated CD and control subjects. This result is in keeping with the demonstration of an increased immunohistochemical expression of GM-CSF in the duodenal mucosa of active CD patients [28].

Recruitment of immune cells into the gut driven by mucosal overexpression of adhesion molecules and chemokines is involved in driving the inflammatory response in CD [13]. In particular, we previously showed that mucosal addressin cell adhesion molecule-1, the ligand of integrin $\alpha_4\beta_7$ expressed on lymphocytes, is increased in untreated CD mucosa, and this couples with depletion of circulating integrin $\alpha_4\beta_7$-positive lymphocytes [29]. As regards chemokines, we reported no difference in terms of CCL1, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL10 and IL-8 between self-reported NCGS patients and controls. Our results are in keeping with those of Brottveit et al. [10] showing unchanged mucosal transcript levels of CCL2 and IL-8 in self-reported NCGS patients after *in vivo* gluten challenge. Moreover, we showed for the first time that CCL1 and CCL4 are released in great quantities by untreated CD biopsies and return to normal after GFD.

We also assessed eosinophil infiltration in self-reported NCGS lamina propria, as eosinophils have been presumed to be implicated in this condition [30]. However, we did not observe their increase in self-reported NCGS lamina propria, this being in keeping with both the recent findings by Zanini et al. [31] and the unchanged IL-5 levels in self-reported NCGS biopsy supernatants. While we acknowledge that these results are not in agreement with those of Carroccio et al. [30], this discrepancy might be related to the different count method used in our study, *i.e.* the peak rather than the number per HPF. The median percentage of IELs was also not significantly increased in self-reported NCGS patients in comparison to controls. Nevertheless, our results, which were obtained in well-oriented duodenal biopsy specimens, are in keeping with those by Brottveit et al. [10] and Bucci et al. [25].

Finally, we evaluated the duodenal biopsies of self-reported NCGS patients for mucosal anti-tTG IgA deposits, which have been proposed as an early marker of celiac immunological response and can be detected in patients with seronegative CD and/or in the absence of intestinal damage on histology [16,32]. The absence of mucosal anti-tTG IgA deposits confirms the absence of an adaptive immunity involvement in self-reported NCGS and makes it possible to exclude cryptic or early forms of CD.

As 11 of the 14 enrolled self-reported NCGS patients subsequently underwent an oral double-blind, placebo-controlled, cross-over gluten challenge trial (for trial description, see Ref. [15]), we had the chance to correlate the mucosal cytokine/chemokine milieu to the clinical response to gluten. This allowed us to neutralize the nocebo effect commonly experienced by subjects who believe they are intolerant to certain foods and widely proved in double-blind, placebo-controlled studies [33–35]. We did not observe any statistically significant correlation between the overall symptomatic (intestinal *plus* extraintestinal) response to gluten – calculated as absolute or differential score – and IEL percentage, lamina propria eosinophil infiltration, cytokines, chemokines or growth factors. Unfortunately, none of the patients included in the present study turned out to be "true" gluten sensitive according to the cut-off value *a priori* established in the double-blind, placebo-controlled, cross-over trial [15]. This drawback together with the lack of an *ex vivo* gliadin challenge of organ culture biopsies do not allow us to exclude an implication of innate immunity in NCGS. However, we believe that understanding the pathogenesis of NCGS cannot disregard the potential role of wheat components other than gluten, such as amylase trypsin inhibitors [8,36], or that of non-immune mechanisms, including the opioid agonistic action of gluten [37–39] or starch malabsorption. Gluten-containing bread and pasta have been shown to cause a significant increase of intestinal fermentation processes even in healthy individuals [40,41], but no data are available in patients with self-reported NCGS. The investigation of these functional mechanisms could provide the pathophysiological explanation for the significant clinical improvement described in NCGS patients following dietary restriction of other wheat components such as fermentable oligo- and disaccharides, monosaccharides and polyols [42].

Conflict of interest

None declared.

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