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# A Functional Idiotype/Anti-Idiotype Network Is Active in Genetically Gluten-Intolerant Individuals Negative for Both Celiac Disease–Related Intestinal Damage and Serum Autoantibodies

**Sara Quaglia,*[,1] Fortunato Ferrara,[†,1] Luigina De Leo,* Fabiana Ziberna,* Serena Vatta,* Serena Marchiò,[‡] Daniele Sblattero,[§] Alessandro Ventura,*[,§] and Tarcisio Not*[,§]**

**An unbalance between Abs that recognize an autoantigen (idiotypes; IDs) and Igs that bind such Abs (anti-IDs) is considered a functional event in autoimmune disorders. We investigated the presence of an ID/anti-ID network in celiac disease (CD), a condition in which antitissue transglutaminase 2 (TG2) Abs are suspected to contribute to CD pathogenesis. To characterize the ID side, we reproduced by in vitro yeast display the intestine-resident Abs from CD and control patients. These TG2-specific IDs were used to identify potential anti-IDs in the serum. We observed elevated titers of anti-IDs in asymptomatic patients with predisposition to CD and demonstrated that anti-ID depletion from the serum restores a detectable humoral response against TG2. Our study provides an alternative approach to quantify CD-related autoantibodies in cases that would be defined "negative serology" with current diagnostic applications. Therefore, we suggest that developments of this technology could be designed for perspective routine tests.** ***The Journal of Immunology*****, 2019, 202: 000–000.**

Each Ab V region is characterized by a peculiar configuration (namely, the idiotype [ID]) responsible for the recognition and binding of a specific target Ag. Igs that recognize these Ag-binding domains are known as anti-ID Abs. They could mimic the Ag itself, of which they are thought to bear an "internal image." Jerne (1) theory predicts that such ID/anti-ID interactions constitute a network involved in the regulation of immunity. By mirroring an original Ag, the anti-IDs can modulate B and/or T cell–mediated responses; in addition, they can maintain B cell memory even in the absence of the Ag (2).

Jerne intriguing hypothesis has been explored in the clinical setting, in which an ID/anti-ID deregulation seems to play a role in autoimmunity, a multifactorial event whose subtended mechanisms are still under exploration. Indeed, in addition to components external to the immune system (e.g., an autoantigen, internal factors), inflammatory reactions and alteration of regulatory mechanisms such as the ID/anti-ID network (3) can trigger an autoimmune response. In healthy individuals, the presence of anti-IDs has been hypothesized to help maintain a nonpathological state by sequestering overexpressed and/or potentially harmful autoantibodies (namely, the IDs) (4). The arise of pathogenic IDs and/or the decrease of protective anti-IDs may unbalance the network and lead to disease onset (5–7). For example, in patients affected by systemic lupus erythematosus (8) or Sjogren syndrome (9), the anti-IDs compete with the autoantigen for binding to circulating autoantibodies, thus modulating their availability and deriving pathogenicity. In type 1 diabetes, Oak et al. (10) reported that Abs against the autoantigen GAD65 are broadly present in the healthy population and that the onset of type 1 diabetes depends on the lack of specific anti-IDs rather than the presence of anti-GAD65 Abs per se. A role for the ID/anti-ID network in autoimmunity has also been evoked in autoimmune thyroiditis (11), Guillain-Barrè syndrome (6), and pemphigus vulgaris (12, 13). Interestingly, the restoration of elevated levels of anti-IDs in patients spontaneously recovering from an autoimmune condition further supports the premise that specific anti-IDs might be of therapeutic significance (3, 14).

Up to 1% of the whole population is affected by celiac disease (CD) (15), making this autoimmune disorder a significant model to investigate the presence of a pathogenic ID/anti-ID network. Several key factors required for the development of CD are known, including the following: 1) genetic predisposition (HLA-DQ2/DQ8

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S.Q., F.F., D.S., S.M., and T.N. designed research, S.Q., F.F., F.Z., S.V., and L.D.L. performed research, contributed reagents, collected data, analyzed and interpreted data, and performed statistical analysis, S.M., F.F., T.N., and A.V. wrote the manuscript.

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Abbreviations used in this article: CD, celiac disease; CGGI, cryptic genetic gluten intolerance; F, female; ID, idiotype; M, male; scFv, single-chain fragment-variable Ab; TG2, type 2 transglutaminase; VH, variable region of the H chain; VL, variable region of the L chain.

allelic variants); 2) external triggers (gluten); 3) self-antigens (type 2 transglutaminase [TG2]); and 4) corresponding autoantibodies (anti-TG2 IgA and IgG) (15). The anti-TG2 Abs isolated in CD patients show unique features, namely 1) their reactivity is restricted to a limited number of epitopes that are shared by all patients (16, 17), and 2) the variable regions of the H chains (VH) are prevalently encoded by the *VH5* gene family (18–20). Production of these autoantibodies is gluten dependent and confined to the intestinal mucosa, as demonstrated by comparing the antigenic responses of peripheral and intestinal lymphocytes recreated by phage display (19) as well as by single-cell sequencing of TG2-reactive mucosal plasma cells (21, 22) and comparative high-throughput sequencing of autoreactive cells in CD patients (20). Recent attention has been directed toward a possible pathogenic effect of the anti-TG2 Abs (23, 24). When tested in vitro in cell models, they alter the enterocyte cytoskeleton and modify cell-cycle dynamics by inducing high proliferation rates, with a pattern similar to the damage at the intestinal villi observed in patients (25, 26). In addition to their primary localization in the small intestine, deposition of these Abs on extracellular TG2 has been observed in several organs (27, 28) in which they can likely contribute to tissue anomalies by inhibiting TG2 enzyme activity and altering extracellular matrix remodeling and cell adhesion. Nonsymptomatic patients at risk for developing CD produce autoantibodies at the intestinal level within the context of a normal mucosa (29, 30). This suggests that intestine-resident anti-TG2 Abs may represent an early marker of genetic intolerance to gluten that can be detected in the absence of typical mucosal lesions (atrophy, intraepithelial lymphocytic infiltrate) and/or circulating markers. We termed this condition "cryptic genetic gluten intolerance" (CGGI) (30).

CGGI individuals are expected to progress to overt CD in the absence of treatment (i.e., gluten-free diet); however, the evolution of CD-related mucosal damages may vary depending on several factors, most of which remain unknown. Although a pathogenic activity of the anti-TG2 Abs is suggested by several studies (25–28, 31), the role of a potential ID/anti-ID network in CD is as yet undetermined. This study was set to investigate both the presence and the function of CD-related IDs and anti-IDs. We found that individuals with a genetic predisposition to CD exhibit elevated levels of circulating anti-ID Igs competent to bind the anti-TG2 Abs and block their interaction with the autoantigen. These results could potentially impact the clinical management of CD.

## Materials and Methods

### *Study subjects*

The subjects of the current study were enrolled at the Institute of Child Health Scientific Institute for Research, Hospitalization and Health Care Burlo Garofolo. All samples were used upon provision of a signed informed consent. The IDs were recreated as yeast-displayed Ab libraries from small intestine mucosal biopsies of 11 patients and 4 healthy subjects (Table I). The presence of anti-IDs was tested in pools of the following sera samples: 25 CD patients (female [F] = 15, male [M] = 1; average age 14.3 [5–28]), 25 CGGI subjects (F = 17, M = 8; average age 21.1 [10–58]), and 25 healthy blood donors (F = 11, M = 14; average age 23.7 [17–50]; 11 HLA-D2 and/or $D8^+$ [including two double positive subjects] and 14 HLA-D2/$D8^-$). The four CGGI subjects whose biopsies served to derive the yeast-displayed Ab libraries were related to the CD patients (three parents and one sibling). To validate the results obtained with pooled sera, we collected individual sera from 10 additional CGGI subjects (F = 7, M = 3; average age 28.8 [12–46]), 10 CD patients (F = 6, M = 4; average age 12 [9–14]), 10 $HLA^+$ healthy subjects (F = 2, M = 8; average age 32 [22–40]), and 10 $HLA^-$ healthy subjects (F = 5, M = 5; average age 29.8 [19–47]). A detailed description of the genetic, clinical, and familial status of all CGGI subjects is reported in Table II.

### *Reproduction of the intestinal anti-TG2 response in yeast-displayed Ab libraries*

The variable region of the L chain (VL) and VH domains of Abs were amplified from cDNAs isolated from intestinal biopsies of the study subjects as previously described (18) using well-characterized primer sets (32). The variable domains were assembled into complete single-chain fragment-variable Abs (scFvs), taking advantage of the yeast homologous recombination mechanism by cotransforming the amplified VH and VL regions with a linearized yeast-display vector into *Saccharomyces cerevisiae*–competent cells (33). In each yeast-displayed library, the total number of independent colonies (corresponding to individual clones) ranged from 5 to 8 $\times$ $10^6$ transformants. The libraries underwent two successive passages of FACS to enrich for TG2 binders as previously described (33) using a FACSAria instrument (Becton Dickinson). Yeast-displayed anti-TG2 clone 2.8 and anti-lysozyme clone D1.3 were produced to be used as positive and negative control Abs, respectively. The Ab copy number on yeast cells (Ab display level) was measured with a PE-conjugated anti-SV5 Ab that recognizes a protein tag fused to the scFv. To evaluate the amount of TG2-reactive Abs, each library was incubated with biotin-conjugated human rTG2 followed by streptavidin–Alexa Fluor 633 conjugate (Thermo Fisher Scientific). Signals were detected with a FACSAria instrument, and data were analyzed with FACSDiva software (Becton Dickinson). Random clones from CGGI libraries were individually validated for their specificity against TG2 with the same protocol using an unrelated Ag (lysozyme) and unconjugated streptavidin as negative controls. Following this analysis, a total of 200 TG2-binding clones were analyzed by Sanger sequencing to characterize the *VH* family gene usage.

### *Characterization of circulating anti-IDs*

The presence of circulating anti-IDs was evaluated in two sets of samples. Serum samples from the first set were combined in the following four pools: a pool of 25 sera from CD patients, a pool of 25 sera from CGGI subjects, a pool of 11 sera from healthy blood donors positive for HLA-DQ2/DQ8, and a final pool of 14 sera from healthy blood donors negative for HLA-DQ2/DQ8. Serum samples from the second set (10 for each of the four study groups) were evaluated individually. Each sample of pooled or individual serum was diluted in PBS at different titers (1:100, 1:200, and 1:400 v/v) and precleared on yeast-displayed anti-lysozyme Ab D1.3 before being incubated with 1 $\times$ $10^7$ yeast cells displaying 1) the anti-TG2 Ab 2.8, 2) each of the 15 libraries, and/or 3) libraries pooled accordingly to the four study groups (CD patient, CGGI subjects, inflammatory disease patients, and healthy individuals; see Table I). The incubations were performed for 2 h at 4°C in rotation. Cells were washed and an APC-conjugated anti-human-IgG Fcγ Ab (Thermo Fisher Scientific) was added at 1:200 (v/v) and incubated for 30 min at room temperature with occasional agitation. Following two washes in PBS, signals were analyzed with a FACSAria instrument and FACSDiva software. The experiments were performed in triplicate.

To capture and deplete the anti-IDs, 200 μl of each precleared serum (pooled or individual) was incubated with either the yeast-displayed anti-TG2 Ab 2.8 or a mix of the CGGI libraries (1 $\times$ $10^6$ yeast cells each) for 1 h at room temperature in rotation. Supernatants were recovered, and sodium azide (0.05% w/v) was added for preservation. The reactivity to TG2 before (whole sera) and after (supernatants) depletion of the anti-IDs was measured by ELISA (Eurospital, Milan, Italy). The experiments were performed in triplicate.

### *Neutralization of circulating anti-TG2 Abs by CGGI-derived anti-IDs*

The anti-IDs were derived from the serum of the same four CGGI subjects whose biopsies had been used for the construction of the yeast Ab display libraries. In a first set of experiments, whole sera were used at different dilutions (1:1, 1:2, and 1:4 v/v). Successive experiments were performed with Ig fractions purified from the same sera (concentrations ranging from 1.5 to 3.6 μg/ml). Purified serum albumin (2 μg/ml) or unrelated Igs (2 μg/ml) were used as negative controls. All these samples were individually incubated with sera from three CD patients at different dilutions (1:1, 1:100, 1:200, 1:400 v/v) at room temperature for 1 h, followed by quantification of anti-TG2 reactivity by ELISA (Eurospital). Ig fractions were purified using the PureProteome Albumin/IgG Depletion Kit (MilliporeSigma), whereas purified albumin (Sigma-Aldrich) and nonspecific Igs (Venital, Kedrion) were obtained commercially.

### *Statistics*

Statistical differences were evaluated by one-way ANOVA followed by Bonferroni posttest and are presented as the mean ± SD of triplicate values. The $p$ values $<0.05$ were considered significant. All statistical analyses were performed with Prism 5 v5.01 (GraphPad Software).

## Results

This work aims to investigate the presence of a functional ID/anti-ID network in CD. A summary of our approach is graphically depicted in Fig. 1. In the first series of experimental procedures, we isolated and characterized CD-related IDs (anti-TG2 Abs) from intestinal lymphocytes by using in vitro yeast-display technology. Successively, these IDs were employed to search for corresponding anti-IDs among the pool of circulating Igs in serum samples. Finally, potential interactions between these two Ab populations and the autoantigen TG2 were evaluated by binding assays as detailed below. These studies were performed on several subjects whose clinical details are listed in Tables I and II.

### *The ID side: individuals at risk for developing CD bear an intestine-resident anti-TG2 reactivity comparable to CD patients*

Fifteen study subjects with different conditions (three CD, four CGGI, four non-CD inflammatory bowel disease, four healthy; details in Table I) were enrolled. Their intestinal Ab repertoire was recreated by in vitro yeast-display technology (33–35). For this purpose, total RNA was extracted from biopsies of the intestinal mucosa of each subject and retrotranscribed in corresponding cDNA. The VHs and VLs were PCR-amplified and inserted by homologous recombination into a yeast-display vector (33) to be expressed on the yeast surface as scFvs (30). The deriving 15 yeast-displayed libraries, therefore, mimic the Ab repertoire of the original intestinal biopsy. These libraries were individually affinity-selected for the isolation of anti-TG2 Abs by two consecutive rounds of FACS with an approach that has been previously validated (33). After this selection step, the ability of each enriched library to recognize the target autoantigen was evaluated in binding assays using human rTG2 and quantified by flow cytometry. Signals were compared with a positive control, namely a clonal yeast population expressing the anti-TG2 Ab 2.8 isolated from a CD patient (36) and a yeast negative control expressing the anti-lysozyme clone D1.3 (Fig. 2A). As expected, a fluorescence intensity analysis confirmed that high-affinity anti-TG2 Abs were present in all the CD libraries with values not significantly different from the positive anti-TG2 control Ab. An anti-TG2 humoral reactivity could be isolated from the CGGI libraries ($p < 0.05$), although with lower values and higher intrasubject variability (Fig. 2B). These results confirm our previous observation of an anti-TG2 response in the intestine of CGGI individuals, which represents a subset of genetic gluten intolerance subjects without intestinal lesions and detectable circulating autoantibodies (clinically defined as "negative serology") (30). Interestingly, a low but positive signal was also observed in libraries from patients with inflammatory bowel diseases, whereas no signal was detected in libraries from healthy subjects (Fig. 2B). Based on previous findings of our group (18, 19) and others (20, 22) describing preferential usage of the *VH5* Ig gene family in CD patients, we also evaluated the *VH* repertoire of the Ab response to TG2 in the CGGI libraries. Two hundred random clones obtained from the CGGI libraries and confirmed as specific for TG2 (data not shown) were Sanger sequenced, revealing that *IGHV5-51*VH* was considerably overrepresented (58% total, of which 41% IGHV5-51*1 F and 17% IGHV5-51*3) compared with all other genotypes (Fig. 2C) and supporting the hypothesis that CGGI is likely an early stage of CD. Together, these data demonstrate that the intestinal autoantibody repertoire of CGGI subjects is quantitatively comparable (presence of reactivity to TG2) and qualitatively similar (*VH5* gene usage) to that observed in CD patients.

### *The anti-ID side: a circulating reactivity to anti-TG2 Abs is peculiarly present in individuals at risk for developing CD*

Having characterized the humoral anti-TG2 response in the intestinal mucosa of different study subjects, we next investigated the presence of circulating anti-IDs (i.e., serum Abs reacting to anti-TG2 Abs) in subjects at risk for developing CD (CGGI) compared with subjects with overt CD or healthy donors. For this set of experiments, four serum pools were obtained by combining 1) 25 sera from CD patients, 2) 25 sera from CGGI subjects, 3) 11 sera from healthy blood donors positive for HLA-DQ2/DQ8 (genetic predisposition to CD in the absence of any other marker), and 4) 14 sera from healthy blood donors negative for HLA-DQ2/DQ8 (no predisposition to CD). These serum pools were initially precleared by incubation with yeast cells displaying the anti-lysozyme Ab to minimize unspecific interactions. Subsequently, each of the four serum pools was incubated with each of the 15 previously described TG2-specific Ab libraries or with the positive control (i.e., yeast-displayed anti-TG2 Ab). After washing, the presence of anti-IDs bound to their corresponding IDs was revealed by flow cytometry, allowing us to quantitatively estimate their reactivity (Fig. 3A). With this procedure, in fact, a fluorescently labeled anti-Fcγ Ab can detect circulating anti-IDs immunocaptured by the yeast-displayed anti-TG2 Abs but not the anti-TG2 themselves as

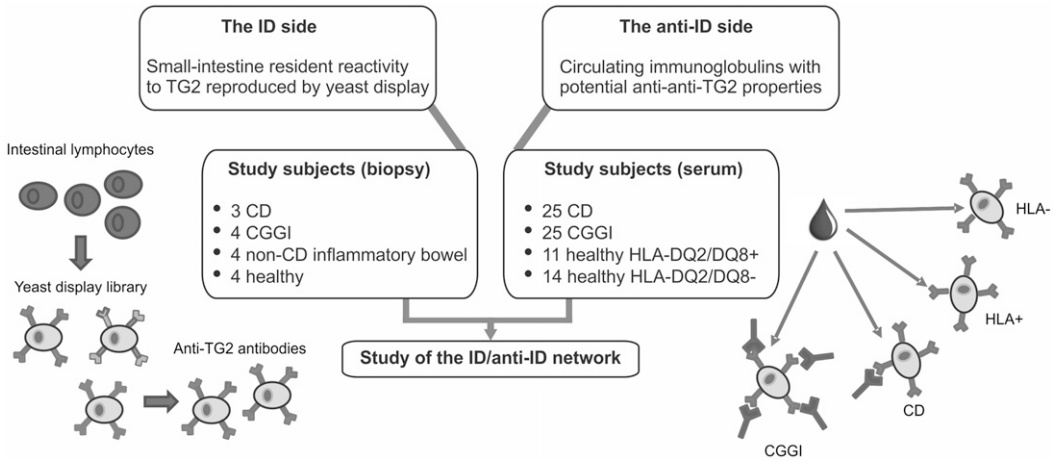


**FIGURE 1.** Schematic representation of the experimental strategy. Small intestine biopsies from several study subjects were used as a source of IDs, whereas sera from another cohort of human subjects provided the potential anti-IDs. The interactions between these two groups of Abs were evaluated as described in the *Results* section.

Table I. Study subjects employed for the yeast library generation

| Study Subject | Sex | HLA | Clinical Presentation | Serum TG2 Reactivity |
|---|---|---|---|---|
| CD | M | DQ2 | Overt CD | pos |
| CD | F | DQ2 | Overt CD | pos |
| CD | F | DQ8 | Overt CD | pos |
| CGGI | F | DQ8 | Asymptomatic | neg |
| CGGI | M | DQ8 | Asymptomatic | neg |
| CGGI | F | DQ2 | Asymptomatic | neg |
| CGGI | M | DQ2 | Asymptomatic | neg |
| Inflammatory bowel disease | M | DQ8 | Non-ulcer dyspepsia | neg |
| Inflammatory bowel disease | M | neg | Chron's disease | neg |
| Inflammatory bowel disease | M | DQ8 | Gastritis from *H. pylori* | neg |
| Inflammatory bowel disease | F | neg | Eosinophilic esophagitis | neg |
| Healthy subject | F | neg | Asymptomatic | neg |
| Healthy subject | F | neg | Asymptomatic | neg |
| Healthy subject | M | neg | Asymptomatic | neg |
| Healthy subject | F | neg | Asymptomatic | neg |

Description of the genetic, clinical, and serological parameters of the study subjects whose intestine-resident humoral immunity has been reproduced by yeast-display Ab libraries.
neg, negative; pos, positive.

they lack the Fcγ portion. In this analysis, sera from CGGI subjects showed a reactivity to all anti-TG2–displaying yeast populations (CD and CGGI libraries, positive control) that was three to four times higher than the reactivity to yeast-displayed Abs from healthy donors, even when the lowest serum titers (1:400 v/v) were challenged. Interestingly, sera from HLA-DQ2/DQ8$^{+}$ healthy subjects also presented a reactivity to anti-TG2 Abs significantly higher than HLA-DQ2/DQ8$^{-}$ healthy subjects, although with a considerably lower intensity compared with CGGI subjects. Based on these promising results, we enrolled other 10 independent subjects from each study group. For this second series of experiments, sera were evaluated individually with the same procedure,

Table II. Detailed data of the CGGI subjects enrolled in the study

| Sex | Age | Relation with CD patient | HLA | Sample | Clinical Presentation | Clinical Outcome After GFD |
|---|---|---|---|---|---|---|
| F | 15 | Sibling | DQ2 | Biopsy and serum | Asymptomatic | Unchanged |
| M | 49 | Parent | DQ8 | Biopsy and serum | Asymptomatic | Unchanged |
| F | 39 | Parent | DQ8 | Biopsy and serum | Asymptomatic | Unchanged |
| M | 53 | Parent | DQ2 | Biopsy and serum | Asymptomatic | Unchanged |
| F | 10 | Sibling | DQ2 | Serum | Asymptomatic | Unchanged |
| F | 44 | Parent | DQ2 | Serum | Asymptomatic | Unchanged |
| F | 56 | Parent | DQ2 | Serum | Asymptomatic | Unchanged |
| M | 39 | Parent | DQ2/DQ2 | Serum | Asymptomatic | Unchanged |
| F | 38 | Parent | DQ2/DQ2 | Serum | Asymptomatic | Unchanged |
| F | 29 | Parent | DQ2 | Serum | Asymptomatic | Unchanged |
| F | 3 | Sibling | DQ2 | Serum | Islet cell Ab positivity | Eliminated |
| F | 10 | Sibling | DQ2 | Serum | Anemia (9 g/dl) | Improved (12 g/dl) |
| F | 14 | Sibling | DQ2 | Serum | Abdominal distension | Improved |
| F | 19 | Sibling | DQ2 | Serum | Abdominal distension | Improved |
| F | 24 | Parent | DQ2 | Serum | Severe constipation | Improved |
| F | 40 | Parent | DQ2 | Serum | Leucopenia, neutropenia, trombocytopenia | Returned to normal values |
| F | 41 | Parent | DQ2 | Serum | Autoimmune thyroiditis | Unchanged |
| M | 41 | Parent | DQ2 | Serum | Anemia (9.8 g/dl) | Improved (12 g/dl) |
| F | 43 | Parent | DQ2 | Serum | Chronic tiredness | Improved |
| M | 43 | Parent | DQ2 | Serum | Explosive diarrhea | Improved |
| M | 43 | Parent | DQ2 | Serum | Abdominal distensions | Improved |
| F | 43 | Parent | DQ2 | Serum | Explosive diarrhea | Improved |
| F | 44 | Parent | DQ2 | Serum | Sjogren disease | Unchanged |
| M | 48 | Parent | DQ2 | Serum | Explosive diarrhea | Improved |
| M | 59 | Parent | DQ2 | Serum | Recurrent elbow arthritis | Improved |
| F | 38 | Parent | DQ2 | Serum | Fatigue | Improved |
| F | 25 | Parent | DQ2 | Serum | Asymptomatic | Unchanged |
| F | 31 | Parent | DQ2 | Serum | Arthritis | Improved |
| M | 30 | Parent | DQ2 | Serum | Asymptomatic | Unchanged |
| F | 18 | Sibling | DQ2 | Serum | Asymptomatic | Unchanged |
| M | 46 | Parent | DQ2 | Serum | Arthritis | Improved |
| F | 30 | Parent | DQ2 | Serum | Explosive diarrhea | Improved |
| M | 15 | Sibling | DQ2 | Serum | Anemia (10 g/dl) | Improved (14 g/dl) |
| F | 12 | Sibling | DQ2 | Serum | Abdominal distension | Improved |
| F | 43 | Parent | DQ2 | Serum | Asymptomatic | Unchanged |

Clinical data of the CGGI subjects enrolled in the study before and after 1 y of gluten-free diet.
GFD, gluten-free diet.

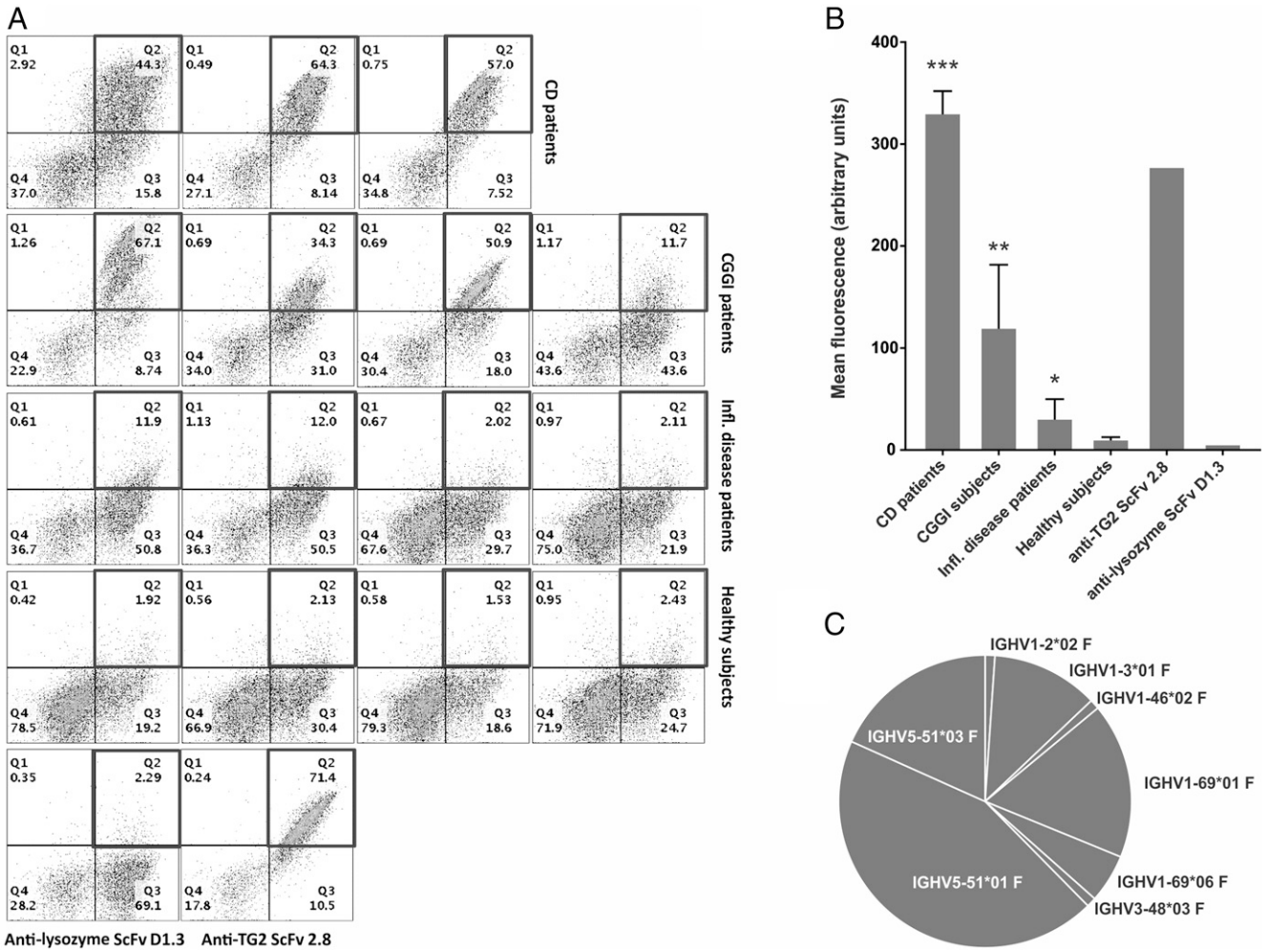


**FIGURE 2.** Recreation and characterization of the intestinal response to TG2 by yeast display. (**A**) Anti-TG2 response recreated on yeast-displayed Ab libraries from biopsies of CD patients ($n = 3$), their asymptomatic relatives (CGGI patients, $n = 4$), patients with CD-unrelated inflammatory bowel diseases ($n = 4$), and healthy subjects ($n = 4$). *x*-axis, Ab display level on yeast cells detected with PE-conjugated anti-SV5 Ab; *y*-axis, positivity to TG2. Yeast-displayed anti-lysozyme Ab D1.3 and anti-TG2 Ab 2.8 were used as a positive and a negative control, respectively. (**B**) Mean fluorescence values for the anti-TG2 reactivity of each study group, evaluated by FACS. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus the negative control. (**C**) VH gene family usage for 200 clones of anti-TG2 Abs from the CGGI group.

confirming the higher reactivity of CGGI subjects to anti-TG2 Abs (Fig. 3B–D), even at the highest serum dilutions (1:400, Fig. 3D), compared with the other study groups. These tests also confirmed the higher reactivity of HLA-DQ2/DQ8$^+$ healthy individuals compared with HLA-DQ2/DQ8$^-$ healthy ones.

These results suggest that specific anti-IDs are present in sera from CGGI and, to some extent, HLA-DQ2/DQ8$^+$ healthy individuals. Moreover, they support perspective diagnostic applications scaled down to individual patients.

### *The ID/anti-ID network: circulating anti-TG2 Abs are sequestered by their corresponding anti-IDs in asymptomatic individuals*

Circulating anti-TG2 Abs are exclusively observed in CD patients, whereas they are not detectable in negative serology settings (30), including individuals with cryptic CD or genetic predisposition to CD (see also Table I). Our above-reported findings suggest that, rather than being absent, such IDs might be sequestered and therefore masked by their corresponding anti-IDs, thus preventing detection with routine diagnostic tools. To verify this hypothesis, we designed an approach in which an excess of IDs is used to deplete potential anti-IDs from the serum and, expectedly, restore a humoral response against the autoantigen (Fig. 4A, schematic representation of the procedure). According to this experimental design, each serum pool was incubated with either the positive control, yeast-displayed anti-TG2 Ab, or a pool of yeast cells displaying the anti-TG2 Abs derived from CGGI patients. After incubation, the yeast-containing fractions were pelleted and discarded, and serum reactivity to human TG2 was evaluated in the supernatants by ELISA. Following the depletion step, no changes were observed in the anti-TG2 reactivity of sera from CD patients (although they all were characterized by the highest reactivity toward TG2) or HLA-DQ2/DQ8$^-$ healthy subjects. In contrast, a dramatic increase in specific signals was observed in sera from CGGI and HLA-DQ2/DQ8$^+$ healthy subjects (Fig. 4B). This increment is even more appreciated when the values are expressed as the ratio of signals detected post- and preincubation (Fig. 4C). As a further step toward potential diagnostic applications of these promising results obtained with serum pools, we applied the same procedure to a patient-focused testing of individual sera. For this new set of experiments, we used sera from a new set of subjects belonging to the same four study groups (CD patients, CGGI subjects, and HLA-DQ2/DQ8$^+$ and HLA-DQ2/DQ8$^-$ healthy donors). After the depletion step, the increased anti-TG2 activity was confirmed, especially in the CGGI subjects,

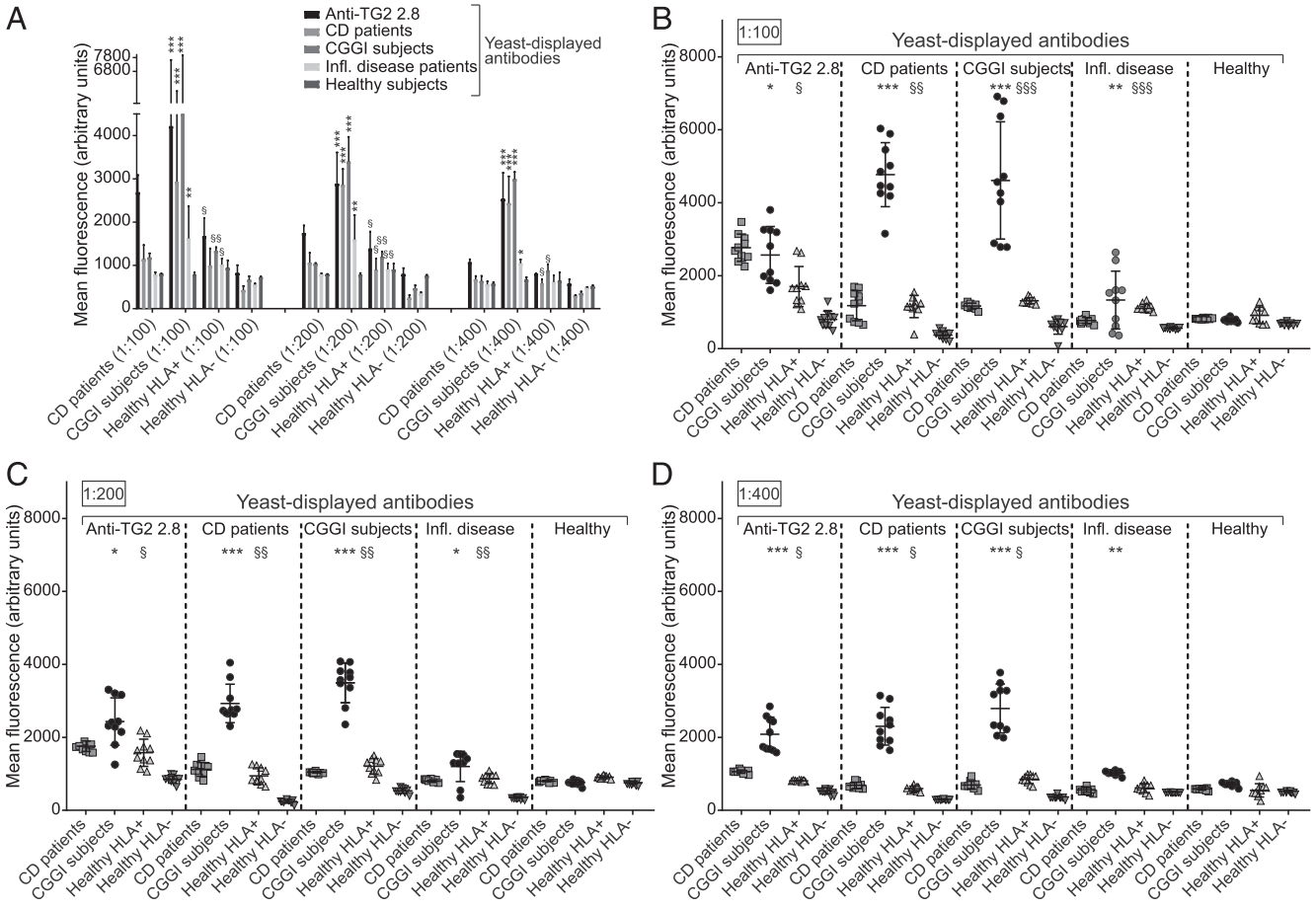


**FIGURE 3.** Identification of serum anti-IDs. (**A**) Different dilutions of each of the four serum pools (overt CD, CGGI, genetic predisposition to CD HLA$^+$, no predisposition to CD HLA$^-$) was precleared on the yeast-displayed anti-lysozyme Ab D1.3, followed by incubation with each of the 15 yeast-displayed libraries or the anti-TG2 Ab 2.8. Bound Igs were revealed by incubation with an APC-conjugated anti-human IgG Fcγ Ab. Signal intensity was measured by FACS. The results obtained with individual libraries belonging to the same group (CD patients, CGGI subjects, healthy HLA$^+$, and healthy HLA$^-$) were combined. Values are shown as mean and SD of repeated readings (positive control) and biological replicates (libraries). (**B**–**D**) show the results of the same experimental approach when single sera were used at different dilutions [(B), 1:100, (C), 1:200; (D), 1:400]. For this second series of experiments, both individual readings and mean/SD of each study group are shown. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, CGGI subjects versus CD patients, HLA$^+$ healthy subject, and HLA$^-$ healthy subjects; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$, HLA$^+$ healthy subjects versus HLA$^-$ healthy subjects.

even at the lowest sera dilution, and such activity was also present in postincubated HLA-DQ2/DQ8$^+$ samples (Fig. 4D). When plotted as a ratio of signal detected post- and pre-incubation, the data clearly show the incremental activity of those sera (Fig. 4E).

These data demonstrate that a coexistence of circulating anti-TG2 Abs and corresponding anti-IDs results in an impaired autoantigen-binding competence that can be restored by depleting the anti-IDs.

### *The ID/anti-ID network: CGGI-derived anti-IDs bind and neutralize the anti-TG2 Abs circulating in CD patients*

As a further step to demonstrate the clinical significance of an ID/anti-ID network in CD, we investigated whether the above-described CGGI-specific anti-IDs could bind circulating anti-TG2 Abs from CD patients and therefore affect their ability to interact with the autoantigen. Initially, sera from four CGGI subjects were serially diluted and individually coincubated with sera from three CD patients selected for the presence of high titers of anti-TG2 Abs (detected by routine testing, data not shown). ELISA quantification of anti-TG2 reactivity was performed before and after incubation. In these assays, incubation with each CGGI serum resulted in a quantity-dependent decrease of the interaction between CD sera and the autoantigen, shown in Fig. 5A as average signal reduction after incubation with each CGGI serum. To rule out if this signal reduction was mediated by unspecific effects dependent on other serum components, the same experiment was repeated with Igs affinity purified from each CGGI serum. Similar to what was observed when assaying whole sera (in which the Ab concentration is not calculated), increasing concentrations of CGGI-derived Igs (1.5, 3 or 6 μg/ml) progressively inhibited the capability of CD sera to bind human rTG2 (Fig. 5B). Although the four CGGI subjects and the three CD patients exhibited different HLA haplotypes (DQ2$^+$ or DQ8$^+$, detailed in Table I), the ELISA outcome was similar for all serum combinations. This result suggests that the haplotype does not correlate with the production of specific anti-IDs, their interaction with the IDs, and/or their capacity to inhibit autoantigen recognition. Finally, to exclude the possibility that either albumin alone (present as contaminant in the purified Ig fraction) or unrelated Abs could affect TG2 binding, we incubated each CD serum with either purified albumin or unrelated Igs (2 μg/ml). In these conditions, the reactivity of CD sera to rTG2 was unchanged (Fig. 5C), confirming the specificity of the observed inhibition. Together, these results demonstrate that binding of the autoantigen by sera of CD patients can be prevented by the addition of anti-IDs derived from asymptomatic individuals.

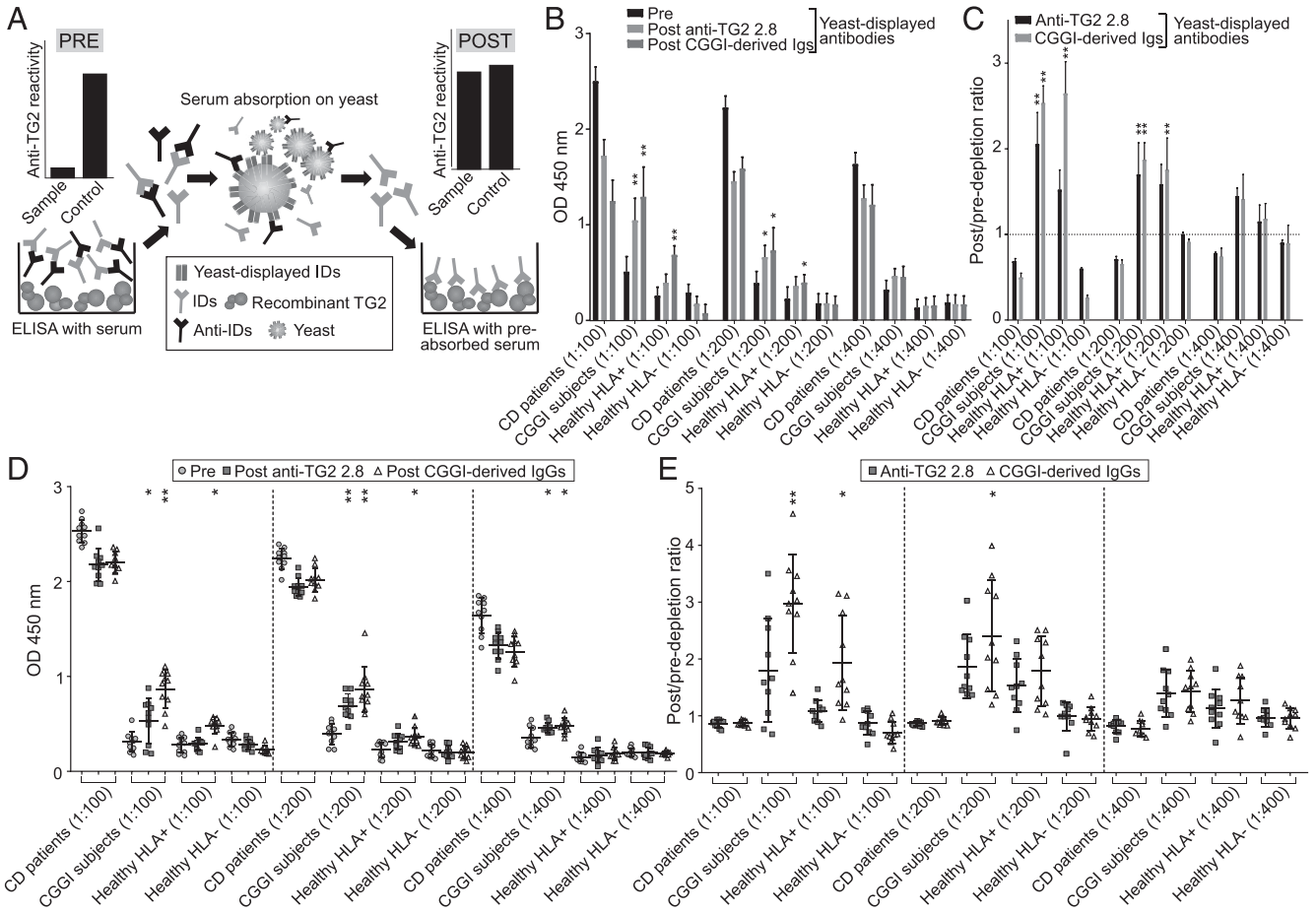


**FIGURE 4.** Depletion of the anti-IDs by incubation with an excess of anti-TG2 Abs. (**A**) Schematic representation of the experimental procedure. The evaluation of anti-TG2 reactivity of the different serum pools was performed by ELISA before and after incubation with yeast-displayed anti-TG2 Abs to remove potential anti-IDs from the serum. (**B**) Each of the four serum pools at different dilutions (overt CD, CGGI, genetic predisposition to CD $HLA^+$, no predisposition to CD $HLA^-$) was incubated with either the yeast-displayed anti-TG2 Ab 2.8 or the mix of the four CGGI libraries, and anti-TG2 Abs were evaluated in the supernatants after discarding the yeast-bound fractions. (**C**) Values are also expressed as the ratio of signals detected post- (supernatants) and preincubation (whole sera) with excess IDs and are shown as mean and SD of three independent experiments. The same analysis was conducted using individual serum samples and shown as row data (**D**) as well as the ratio of signals detected post- and preincubation with excess IDs (**E**). For this series of experiments, both individual readings and mean/SD of each study group are shown. In (B) and (D), $*p < 0.05$, $**p < 0.01$ increased versus preincubation; in (C) and (E), $*p < 0.05$, $**p < 0.01$ versus the reference value set at 1.

## Discussion

In this study, we investigate if the presence of an ID/anti-ID network is the mechanism responsible for protecting genetically predisposed individuals from developing overt CD.

In particular, CD presents itself as an interesting disease model, because several components of CD onset are well defined, including genetic predisposition, role of gluten in triggering an autoimmune response, and Ags targeted by the autoantibodies (15). In point of fact, several experimental evidences have focused on the possible pathogenic role of anti-TG2 autoantibodies because of their interaction with TG2 (23, 24). However, it remains partially unclear why some people do not progress despite producing high amounts of anti-TG2 Abs (29, 30), which are suspected to contribute to the mucosal damage observed in patients with overt CD (25–28, 31). Moreover, although these individuals, when on a gluten-containing diet, show an intense autoantibody production at the intestinal level, they usually test negative for anti-TG2 reactivity at serological evaluation. Together, these observations suggest that, in some cases, the autoantibodies may be sequestered or otherwise blocked/neutralized. As observed in other autoimmune conditions, an active ID/anti-ID network could explain these anomalies (3).

We reproduced the repertoire of CD-related intestinal IDs (anti-TG2 Abs) by yeast display. The yeast libraries, enriched for anti-TG2 Abs, were challenged with serum samples as a source of anti-IDs (anti-anti-TG2 Abs). Yeast Ab libraries displaying CD-specific IDs can be employed to evaluate the presence of corresponding anti-IDs because Abs binding to anti-TG2 Ig displayed on the yeast cells can be easily recovered by centrifugation and successively quantified by flow cytometry.

This analysis was first focused on serum pools to maximize the possibility to detect an anti-TG2 (anti-ID) response, also considering that each CGGI patient might have a peculiar anti-ID–binding profile, recognizing different epitopes of the anti-TG2 Abs. However, this approach has limitations, including the impossibility to determine if the detected signal is due to one (or very few) sample(s) containing Abs specifically recognizing anti-TG2 (private ID) or if the measured activity is due to the contribution of more common anti-IDs shared among CGGI individuals (public ID). For this reason, after proving a significant activity toward anti-TG2 Abs when pooled sera were used, a different set of individual sera was used to confirm the results.

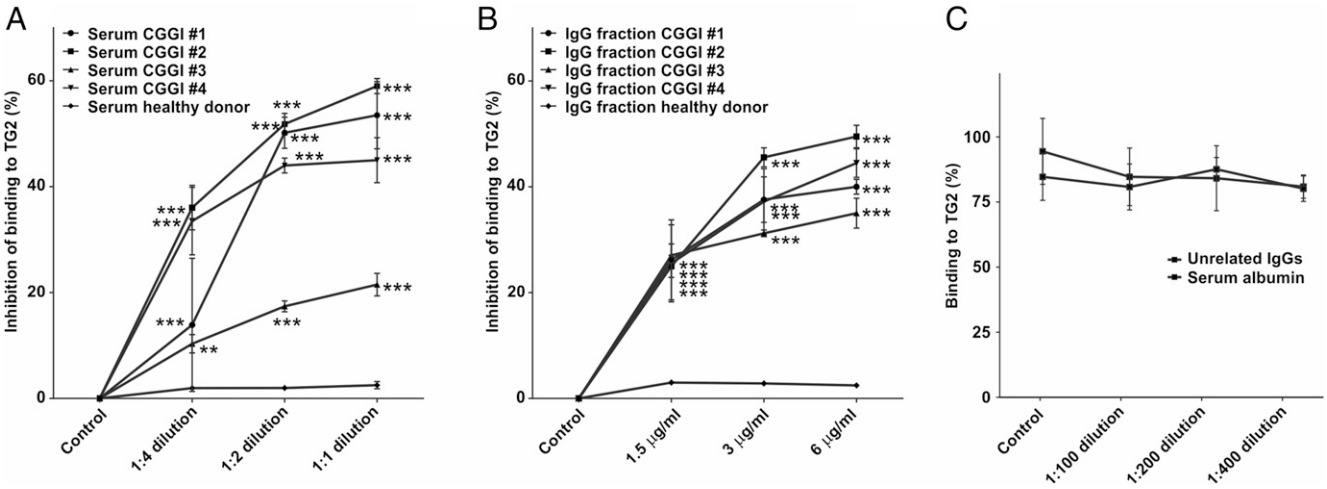


**FIGURE 5.** Block of circulating CD autoantibodies by addition of CGGI-derived anti-IDs. (**A**) Scalar dilutions of four CGGI sera (1:1, 1:2, and 1:4 v/v in PBS) were incubated with sera from three CD patients (1:200 v/v in PBS), followed by evaluation of anti-TG2 activity by ELISA. (**B**) The same experiment was repeated with CGGI sera-derived Igs at scalar concentrations (1.5, 3 or 6 μg/ml in PBS). In all cases, values are represented as percentage of binding to TG2 compared with untreated sera and are shown as mean and SD of the three experimental points. $^{**}p < 0.01$, $^{***}p < 0.001$ versus healthy donor. (**C**) Scalar dilutions of each CD serum (1:1, 1:100, 1:200, and 1:400 v/v in PBS) were also incubated with either purified serum albumin or unrelated Igs (1.5, 3 or 6 μg/ml in PBS), and values are represented as percentage of the binding activity of the sera.

With this approach, we demonstrated that circulating anti-IDs are specifically present with a high titer in individuals with cryptic CD (CGGI) and, to a lesser extent, with a mere genetic predisposition to CD (HLA-DQ2/DQ8$^+$), especially when compared with HLA-DQ2/DQ8$^-$ subjects. The presence of a detectable humoral response to anti-TG2 Abs in CGGI can be considered in relation to a potential protective role in patients who, despite showing extensive anti-TG2 deposits at the intestinal level (30), do not experience CD-related symptoms. Interestingly, the reactivity above the threshold present in sera of healthy donors with only genetic predisposition to CD (HLA-DQ2/DQ8$^+$) suggested that the anti-ID network might play a protective role at the very early stages of disease onset, although CD patients lack anti-ID Abs and their potential inhibitory function.

After demonstrating the possibility to detect Igs binding to anti-TG2 Abs in particular subsets, we wanted to investigate their nature as true anti-ID and their specificity by depleting them from the serum by an excess of their interacting IDs. This was achieved, again, exploiting yeast-displayed anti-TG2 Abs as a source of IDs, considering that as many as 20,000–100,000 Ab copies are exposed on the surface of each yeast cell (35). Our results demonstrate that a coexistence of circulating anti-TG2 Abs and corresponding anti-IDs, peculiar to individuals with cryptic CD or genetic predisposition to CD, results in an impaired autoantigen-binding competence that can be restored by depleting the anti-IDs. This condition is uniquely associated with asymptomatic individuals (as opposed to patients with overt CD), further corroborating the hypothesis that an ID/anti-ID network might have a functional role in preventing or delaying CD onset and/or progression. These results were achieved by using both pooled and individual sera, confirming that the detected idiotypic network is not because of few predominant samples with higher anti-ID titer (a restricted private ID), but that it is a phenomenon shared among the individuals characterized as CGGI (public ID).

The subsequent step consisted of directly evaluating and demonstrating that the sera and the Igs of individuals with a genetic predisposition to CD and with possibly elevated levels of circulating anti-IDs, are able to bind the anti-TG2 Abs and block their interaction with the autoantigen. A first consequence of this observation is that, in these genetically predisposed individuals, the reactivity to TG2 is masked by the anti-IDs and can lead to negative results at serological evaluation; for this reason, the depletion of anti-IDs could be useful in the design of novel diagnostic tests to identify individuals at risk for developing overt CD. Interestingly, our data suggest that the haplotype does not affect the anti-TG2 reactivity in CGGI subjects, at least in this pilot investigation on a small cohort of patients, so that the described approach could likely be applied to a broad panel of individuals at risk for developing CD. Based on these promising results, we are planning to extend the investigation to a larger cohort that would also reproduce the typical distribution of HLA haplotypes, in which DQ8$^+$ individuals contribute to 5–10% only of all CD patients. This will allow a more comprehensive study to rule out potential correlations/interferences between the haplotype and a correct autoantibody detection. A second aspect is that the high levels of anti-IDs in the sera of genetically predisposed individuals could postpone, or even block, the emergence or the activity of potentially harmful IDs. Considering the reported pathological effect of the "anti-TG2 Ab/TG2" molecular axis (25–28, 31), a potential implication of our findings could be that future development of therapeutic tools based on purified anti-IDs might be conceived to delay CD progression. In support of this hypothesis, in a previous study, we observed a significant correlation between an elevated level of anti-IDs and a reduction of tissue lesions in an animal model induced to produce anti-TG2 Abs (37).

In the current study, we have started to investigate an immunoregulatory mechanism within the context of CD. In future work, we plan to better define the nature of the factors that interfere with anti-TG2 reactivity. This may help in the optimization of autoantibody-based assays in patients at high risk of developing CD in which the serum anti-TG2 Abs are masked by high titers of anti-IDs, thus leading to false negative quantifications. Undoubtedly, measuring the presence and amounts of circulating anti-IDs during the shift from CGGI to overt CD will be essential to understand how the autoimmune responses are regulated in disease progression. Moreover, because the idiotypic network likely plays a nonsecondary role in controlling the progression of CD by neutralizing the potential harmful Abs or by downregulating their secretion (as observed also in other autoimmune diseases), modulating and controlling such networks may offer alternative

therapeutic possibilities. For this reason, CGGI-derived anti-IDs could represent innovative tools to block the activity of anti-TG2 Abs in CD patients, thus reverting their pathogenic activity (23, 24).

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## Disclosures

The authors have no financial conflicts of interest.

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