Distinct Anti-IFI16 and Anti-GP2 Antibodies in Inflammatory Bowel Disease and Their Variation with Infliximab Therapy

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Conflicts of Interest and Source of Funding

Dr. Dirk Roggenbuck is shareholder of GA Generic Assays GmbH and he has patents relevant to the work. The remaining authors have no conflict of interest to disclose

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ABSTRACT

Background: Inflammatory bowel disease (IBD) is characterized by a chronic inflammation of the gut, partly driven by defects in the expression and function of pattern recognition receptors (PRR), including the IFI16 protein. Since this protein is a target for autoantibodies and its aberrant expression was reported in colonic mucosa from active UC patients, we studied its expression and specific seroresponse in IBD patients before and after infliximab (IFX) therapy.

Methods: Anti-IFI16 antibodies (IgG and IgA subtypes) were measured in the sera of 74 IBD patients: 48 Crohn's disease (CD) and 26 ulcerative colitis (UC) patients, prospectively harvested before and after IFX therapy. Anti-GP2 antibodies (both IgG and IgA subtypes) were also tested for comparison. The patient antibody statuses were qualitatively and quantitatively associated with disease phenotype and response to IFX therapy.

Results: Significantly higher titres of anti-IFI16 IgG were found in both CD and UC patients compared with healthy controls (HC). Anti-IFI16 IgA titres were also present in CD patients. Anti-GP2 IgG subtype titres were significantly increased in CD patients, as were IgA subtype titres. Significant changes in anti-IFI16 IgG subtype titres were observed after IFX in CD patients that correlated with clinical remission or response.

Conclusions: Our results highlight the importance of IFI16 in IBD pathogenesis showing that its *de novo* overexpression in the gut epithelial cells leads to a breakdown in immune tolerance and the subsequent development of specific autoantibodies. Anti-IFI16 IgG antibodies hold the potential to serve as a biomarker of response to IFX therapy.

Keywords: IFI16, Crohn's disease, autoantibodies

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic, immune-mediated inflammatory disorders of the gastrointestinal system, including Crohn's disease (CD) and ulcerative colitis (UC), that fluctuate between active and inactive phases of the disease.[1-4] IBD has a multifactorial aetiology with a number of gene polymorphisms conferring susceptibility, and environmental triggers leading to disease onset. It has been associated with changes in the intestinal microflora, defects in the microbiological and physical intestinal barrier function with increased penetrance of commensal microorganisms and other luminal content into the mucosal layer, and a loss of immune tolerance.[1, 5-8] Consequently, specific adaptive immune responses towards luminal antigens are altered in IBD patients.[1, 9]

Recent studies into IBD pathogenesis have shone light onto key disease mechanisms, including the innate and adaptive immune responses and the interactions between genetic factors and microbial and environmental cues.[1, 10,11] Significant alterations in the behaviour of cells mediating innate immunity and in the expression and function of pattern recognition receptors (PRR), including TLRs and PYHIN-200 that recognize microbial antigens, were recently described in IBD.[12-19] Indeed, mice deficient in PRR exhibited increased susceptibility to different experimental models of colitis.[7, 20-23]

The PYHIN200 family encodes evolutionary-related human (IFI16, IFIX, MNDA, AIM2) and murine (Ifi202a, Ifi202b, Ifi203, Ifi204, Ifi205/D3 and ifi206) proteins.[24] Two members of the human family, IFI16 and AIM2, have been implicated in the recognition of pathogen DNA and classified into the ALR (AIM2-like receptor) group. Upon activation by pathogen DNA, IFI16 translocates into the cytoplasm, triggers type I IFN production, inflammasome expression, and induces the release of inflammatory cytokines, including IL-1β and IL-18, and eventually cell death.[25-28] IFI16 is then released into the extracellular milieu where it behaves as an alarmin, contributing to the progression of inflammation and autoimmunity.[29] Reports from our group and other laboratories have revealed the role of these proteins in the development of autoimmune

diseases. We have shown that the IFI16 protein is a target for autoantibodies. The presence of anti-IFI16 antibodies has indeed been demonstrated in patients with Systemic Lupus Erythematosus (SLE), Sjogren Syndrome (SjS), Systemic Sclerosis (SSc) and Rheumatoid Arthritis (RA).[30, 31] The appearance of anti-IFI16 antibodies is more likely to occur in the slowly progressive and limited cutaneous setting of SSc, while in SLE they are associated with a lower risk of developing proteinuria.[32-34] Aberrant IFI16 expression has also been demonstrated in minor salivary glands from patients with primary SjS.[35]

A recent report by Wiebe Vanhove highlighted the importance of PYHIN inflammasome signalling in IBD and linked the responsiveness of anti-tumour necrosis factor (anti-TNF) therapy to signalling mediated by these inflammasomes.[12] They demonstrated a significant upregulation of AIM2 and IFI16 proteins in colonic mucosal biopsies from active UC patients versus controls. Moreover, responders to anti-TNF therapy showed lower expression levels of these inflammasomes, although IFI16 remained significantly higher in responders showing endoscopic healing versus controls.

In this study, we investigated the presence of the IFI16 protein in mucosal biopsies from patients with IBD and the presence of anti-IFI16 autoantibodies (IgG or IgA) in their sera, and compared them with healthy controls.

The levels of anti-glycoprotein 2 (GP2) autoantibodies were also analysed as they have been reported to be present in up to 40% of the patients with CD and can therefore be used in some cases to differentiate CD from UC, in which anti-GP2 are absent.[36-38] Furthermore, a growing body of evidence suggests that the presence of anti-GP2 Abs is associated with specific disease phenotypes, thus their detection may be of clinical significance.[39-42] GP2 is found in acinar cells of the exocrine pancreas and reported to be shed, together with digestive enzymes, into the pancreatic duct and transported into the intestinal lumen where it can opsonize FimH-positive microbes (FimH+).[43] GP2 is also synthesized in microfold cells (M) of the follicle-associated epithelium, where it is presented as a membrane bound-receptor that can grab FimH-positive bacteria for transcytosis by these

cells.[44]

The aim of this longitudinal study was to assess the performance of anti-IFI16 autoantibodies, alone or in combination with anti-GP2, in the diagnosis of IBD, disease stratification and disease phenotype, and to assess whether antibody status could be qualitatively and quantitatively associated with a patient's response to infliximab (IFX) therapy. This study demonstrates, for the first time, the presence of anti-IFI16 IgG and IgA antibodies in the serum of IBD patients and confirms the presence of anti-GP2 IgG and IgA in CD. We also detected marked changes in the anti-IFI16 immune response after induction therapy with IFX, especially in CD patients.

MATERIALS AND METHODS

Patients and samples

After obtaining written informed consent, consecutive IBD patients scheduled to undergo Infliximab (IFX) therapy at the Gastroenterology and Digestive Endoscopy Unit of IRCCS Policlinico San Donato between June 2008 and August 2014 were prospectively enrolled onto the study. All diagnoses had been previously confirmed by clinical, endoscopic and histological criteria. The indications to start IFX treatment were steroid-dependent/refractory disease and/or the presence of perianal disease, according to current guidelines.[45-47] IFX therapy (5 mg/kg/infusion) was performed according to a standard induction (T_0, week 2 and week 6) and maintenance schedule (every 8 weeks) in all cases. Seventy-four IBD patients, 48 CD (25 men and 23 women, mean age 41 years; range 16-65 years) and 26 UC (21 men and 5 women, mean age 39 years; range 17-62 years), were included in this study.

Serum samples were prospectively collected just before the initiation of the first Infliximab infusion (IFX1 or baseline) and from then on immediately before every IFX infusion during maintenance therapy (Fig. 4A). Samples were collected and analysed blind to clinical data and stored at -20°C. C-reactive protein (CRP) levels were also measured at every IFX infusion.

As part of the enrolment process, disease activity was assessed by means of endoscopical evaluation and through the use of clinical disease activity scores: the Harvey-Bradshaw Index (HBI) was used for CD patients[48] and the Mayo score was used for UC patients;[49] disease activity was defined as moderate for a HBI score < 5 and severe for a HBI score ≥ 5, and as mild for a Mayo score ≤ 4 and moderate for and a Mayo score > 4. The HBI score was calculated at the time of each infusion in CD patients; whereas in UC patients a partial Mayo score (calculated in the absence of the endoscopic sub-score) was used to monitor clinical activity at the time of each drug administration. In CD patients, response to Infliximab therapy was defined as: clinical remission = HBI < 4; clinical response = a decrease of ≥ 3 points in the HBI score compared to the previously obtained score. In UC patients, response to Infliximab therapy was defined as: clinical remission = partial Mayo score ≤ 2 with no individual subscore > 1; clinical response = a decrease of ≥ 3 points in the Mayo partial score and at least 30% from the baseline score, with a decrease in the rectal bleeding subscore of ≥ 1 point or an absolute rectal bleeding subscore of 1 or 0.

The demographic, clinical and laboratory data obtained at the time of the first blood sampling are reported in Table 1. Sera from 182 sex- and age-matched healthy controls (HCs) were recruited from the blood bank in Novara.

Colon biopsies from 5 UC patients were taken from macroscopically inflamed areas during colonoscopy procedures. For CD, tissue sections from 7 different surgical specimens were used. Control sections corresponded to mucosa of normal appearance, obtained from gut resection for cancer. Sections from infectious colitis, ischemic colitis, and diverticulitis (n=3 each) were also investigated as inflammatory non-IBD controls. None of these tissues were from the study cohort.

Written informed consent was obtained from all participants according to the Declaration of Helsinki, and approval of the study protocol was obtained from the local ethics committees.

Immunohistochemistry

Sections from formalin-fixed and paraffin-embedded specimens (5μm thick) were processed as

previously described.[50] As negative controls, appropriate slides were incubated with PBS instead of primary antibodies and underwent the same staining procedure.

Determination of antibody titres towards human recombinant IFI16 and GP2 by ELISA

To detect anti-IFI16 IgG antibodies, polystyrene micro-well plates (Nunc-Immuno MaxiSorp; Nunc, Roskilde, Denmark) were coated with a solution of recombinant IFI16 in PBS and, after blocking, sera were added in duplicate, as previously described.[32]

To detect anti-IFI16 IgA, polystyrene micro-well plates were coated with a solution of recombinant IFI16 in PBS. After blocking, sera were added in duplicate. After washing, horseradish peroxidase-conjugated rabbit anti-human IgA (Dako, Cytomation, Carpinteria, CA, USA) was added. Following the addition of the substrate (TMB, KPL, Gaithersburg, MD, USA), absorbance was measured at 450 nm using a microplate reader (SpectraCount, Packard, Packard BioScience Company). The background reactivity of the reference mixture was subtracted to calculate the results. A standard curve was constructed by serially diluting purified IgA from a positive patient,

Glycoprotein 2 autoantibodies were detected in the sera of patients and controls using ELISA employing recombinant human GP2 isoform 4 as the solid-phase antigen (anti-GP2 IgA and anti-GP2 IgG ELISA [GA Generic Assays, Dahlewitz/Berlin, Germany]) according to the manufacturer's instructions.[37, 38]

Statistical analysis

Data are expressed as mean ± standard deviations (SD), median and interquartile ranges (IQR), absolute values, percentages or percentiles. Positivity cut-off values for anti-IFI16 IgG and IgA antibodies were calculated as the 95th percentile of the control population. Positivity cut-off values for anti-GP2 IgG and IgA are predetermined by the manufacturers. The Mann-Whitney test, Wilcoxon signed-rank test, Fisher's exact test or Chi-square test were used. Multiple logistic regression was used to determine the association of anti-IF16 and anti-GP2 IgG and IGA with the

odd ratio (OR, 95% CI) of clinical parameters (location, behaviour, response to therapy). Due to the relative low number of patients, clinical response and clinical remission (in both CD and UC patients) and behaviours B2 and B3 in CD patients were considered as a single variable. Statistical significance was assumed at $p < 0.05$. Statistical analysis was performed using SPSS 21.0 (SPSS Inc, Chicago, IL).

RESULTS

Aberrant IFI16 expression is occurring in the intestinal epithelial cells of IBD patients and is associated with elevated levels of anti-IFI16 autoantibody

In the normal setting, the IFI16 protein is only detectable in haematopoietic cells, endothelial cells and keratinocytes. The mucosal epithelium of the small and large bowel is negative for IFI16 protein expression in healthy individuals;[50] however, Vanhove et al. recently reported that IFI16 expression is strongly upregulated in gut epithelial cells in patients with active UC compared to healthy controls.[12]

To gain more insight into IFI16 expression in the intestinal mucosa from IBD patients, intestinal samples from both UC and CD cases were analysed by immunohistochemistry using anti-IFI16 antibodies. As shown in the representative images reported in Figure 1, colonic expression levels are low in the normal setting (healthy controls) and restricted to endothelial and inflammatory cells in the intestinal lamina propria (LP), with a clear nuclear localization (Fig. 1, left panels). A very different picture emerges in the samples from both CD and UC patients (Fig. 1, middle and right panels, respectively), where the colonic expression of IFI16 protein is substantially higher and well evident also in the nuclei of the epithelial cells. IFI16 expression in intestinal epithelial cells is specifically occurring in IBD as demonstrated by the lack of staining in other non-IBD inflammatory diseases used as control, such as ischemic colitis, diverticulitis and infectious colitis. Despite a very strong stromal reactivity, no IFI16 staining was detected in colonic epithelial cells in

these settings (data not shown).

Altogether, these findings support the hypothesis that IBD-related inflammation is accompanied by IFI16 *de novo* overexpression in the gut mucosal cells from IBD patients, which could then lead to a breakdown in immune tolerance to the IFI16 protein, resulting in the development of specific autoantibodies. To verify this hypothesis, we tested the sera of 74 IBD patients (48 CD and 26 UC patients), harvested prior to the start of IFX therapy, for the presence of anti-IFI16 antibodies by ELISA (both IgG and IgA subtypes). Anti-GP2 antibodies (both IgG and IgA subtypes) were also tested for comparison. The characteristics of the patients included in the study are reported in Table 1, including their response to IFX therapy, as determined after induction therapy, before the fourth IFX administration (IFX4).

Higher anti-IFI16 IgG titres were detected in CD [59.0 U/ml (IQR, 41-79)] and UC [67.2 U/ml (IQR, 44-165)] patients compared with healthy controls [HC: 25.9 U/ml (IQR, 17-45), $p<0.0001$]. With cut-off levels corresponding to the 95th percentile of the distribution in the control population (113 U/ml), 19% of CD and 31% of UC tested positive for anti-IFI16 IgG autoantibodies in comparison with 6% of HC ($p=0.0171$ and $p=0.0006$, respectively) (Fig. 2A, left panel). The sensitivity and specificity of these cut-off levels have been previously validated with a receiver operating characteristics (ROC) curve.[33] Median anti-IFI16 IgA titres tended to be higher in CD [4.1 U/ml (IQR, 3-6)] compared with UC [3.5 U/ml (IQR, 3-5), $p=0.1948$] and HC [3.7 U/ml (IQR, 2-5), $p=0.0748$], although statistical significance was not achieved. However, using a cut-off threshold corresponding to the 95th percentile of the distribution in the control population (9.6 U/ml), 17% of CD patients tested positive for antibodies of the IgA subtype in comparison with just 4% in the UC population and 5% in HC (CD *vs*. HC $p=0.0258$) (Fig. 2A, right panel).

The same sera were also quantitatively tested for the presence of anti-GP2 autoantibodies (IgG and IgA subtype) by ELISA. As expected, significantly higher anti-GP2 IgG titres were observed in CD patients compared to UC [median levels: 8.2 U/ml (IQR, 0-19) *vs*. 0.0 U/ml (IQR, 0-5), respectively, $p=0.0026$] and HC [4.9 U/ml (IQR, 3-10), $p=0.3340$]. Using a cut-off of 15 U/ml

(determined by the manufacturer), 29% of CD patients tested positive for anti-GP2 IgG in comparison with 8% of UC patients (p=0.0398) and 14% of HC (p=0.0274) (Fig. 2B, left panel). A similar pattern was also observed for the anti-GP2 IgA subtype, even if the difference between median levels of CD compared to UC and HC did not reach statistical significance [2.5 U/ml (IQR, 0-6), 2.4 U/ml (IQR, 0-4) and 1.8 U/ml (IQR, 1-3), respectively]. However, considering a cut-off of 10 U/ml, IgA antibodies were present in 15% of patients with CD and 12% of those with UC in comparison with 2% of HC (CD *vs*. HC p=0.0033) (Fig. 2B, right panel).

Correlations of anti-IFI16 and anti-GP2 antibody levels with patient features

Next, we tested the relationship between positivity of specific seroreactivity with the demographical and clinical parameters reported in Table 1 by means of logistic regression analysis. For this analysis, clinical response and clinical remission were combined to form a single group in both CD and UC patients. Moreover, in CD patients, B2 and B3 behaviours were also combined into to form single group due to the low number of samples. No significant associations were found between the four types of autoantibody tested at baseline and patient gender or age. The occurrence of anti-IFI16 isotypes IgG and IgA did not differ between patients with a moderate (HBI < 5; 22% and 11% respectively) vs. severe disease state (HBI ≥ 5; 18% and 20%, respectively). Similarly, no correlation existed between the presence of anti-IFI16 autoantibodies and clinically active UC (data not shown). Furthermore, the prevalence of anti-IFI16 was similar between CD and UC patients with C-reactive protein (CRP) levels ≤1 mg/l (16% in CD and 21% in UC for the IgG subtype, and 24% in CD and 7% in UC for the IgA subtype) and those with levels > 1 mg/l (22% in CD and 42% in UC for the IgG subtype, and 9% in CD and 0% in UC for the IgA subtype). When we analysed the correlations between the presence of the autoantibodies tested and disease location, behaviour and response to induction therapy with IFX in CD patients, several statistically significant associations were found (Table 2). Positivity for anti-IFI16 IgA was associated with a higher risk of colonic localization (L2) (OR=7.789; 95% CI 2.144-28.354), and positivity for anti-GP2 IgG were

associated, as expected, with ileocolonic localization (L3) (OR=5.542; 95% CI 1.993-15.411). Likewise, elevated titres of the IgA isotype were associated with ileal (L1) (OR=9.852; 95% CI 1.456-66.684) and ileocolonic localization (L3) (OR=11.822; 95% CI 2.413-57.932). By contrast, we did not find any association of anti-IFI16 IgG with disease location in CD patients. In addition, analysis of disease behaviour revealed that positivity for anti-IFI16 IgG was associated with stricturing or penetrating (B2 or B3) disease behaviour (OR=4.275; 95% CI 1.025-17.829), while the IgA isotype was associated with the presence of perianal manifestations (OR=5.347; 95% CI 1.637-17.466). Elevated levels of anti-GP2 IgG were indeed associated with the more benign form of the disease, defined as presenting non-stricturing and non-penetrating behaviour (B1) (OR=6.158; 95% CI 1.627-23.307). Surprisingly, positivity for anti-GP2 IgA was associated with all behavioural patterns using healthy control as reference (B1: OR=11.083; 95% CI 1.615-76.070; B2 or B3: OR=9.852; 95% CI 1.456-66.684; B4: OR=5.542; 95% CI 1.055-29.095). By contrast, when we performed the same analysis using patients with non-stricturing and non-penetrating behaviour as reference, as reported by Papp et al.,[40] we failed to detect any significant association very likely due to the limited number of patients in our cohort.

Patients with low levels of IgG antibodies against IFI16 at baseline had a higher probability of a clinical response or remission at the end of induction therapy with Infliximab (OR=0.143; 95% CI 0.027-0.761).

The only statistically significant association found in UC patients was that between anti-IFI16 IgG and extensive colitis (E3) (OR=7.255; 95% CI 2.452-21.463; p=0.0001) (data not shown).

Anti-GP2 IgG and anti-GP2 IgA titres were positively associated in CD patients (R=0.933, p = 0.0001). By contrast, no significant correlations were found between the other antibodies in either the CD or the UC population (Fig. 3).

Evaluation of anti-IFI16 and anti-GP2 autoantibody patterns in IBD patient sera before and after infliximab therapy

The effect of anti-inflammatory treatment with IFX on the levels of all four serological markers (anti-IFI16 IgG and IgA and anti-GP2 IgG and IgA) was studied in the patient population throughout the observation period (schematically represented in Figure 4A). After the first infusion (IFX1), 391 serum samples were collected during follow up, with an average of 8 samples per patient. All the samples were tested for the anti-IFI16 antibodies; while for anti-GP2 antibodies, only the sera corresponding to IFX1 and IFX3 were tested. At the end of the induction period (IFX4), 63% of patients (47/74) achieved clinical remission, 13% (10/74) achieved clinical response, and 23% (17/74) failed to respond to induction therapy. To increase the statistical power of the analysis, autoantibody levels in sera sampled at IFX3 were compared with IFX1 because all the study patients were sampled at the IFX3 time point.

In the CD patients, titres of the anti-IFI16 IgA subtype did not change over the study session. On the other hand, anti-IFI16 IgG titres were significantly higher at IFX3 than at IFX1 [80.2 U/ml (IQR, 53-180) *vs*. 59.0 U/ml (IQR, 41-79), p=0.0002] (Fig. 4B, left panel) in the CD population. In the same patients, anti-GP2 IgA, but not IgG, were higher at IFX3 than IFX1 [4.1 U/ml (IQR, 1-7) *vs*. 2.5 U/ml (IQR, 0-6), p=0.0011] (Fig. 4C, right panel). In UC patients, the prevalence of the anti-IFI16 IgG subtype at IFX3 was 54% (compared with 31% at IFX1, not statistically significant p=0.1599) and 0% for the IgA subtype (4% at IFX1, not statistically significant p=1.0000). No significant changes were detected in the levels of the anti-IFI16 autoantibodies (both subtypes) at the later time points in the whole cohort and the marker status of each individual patients did not change over time.

It is worth noting that when the CD patients were grouped based on the increase or decrease of anti-IFI16 IgG titres at the IFX3 time point, we found out that the majority of those with clinical response or remission showed a rise in autoantibodies against IFI16 (p=0.05 Pearson's chi-squared test). In those who were considered to be in clinical remission at IFX3 (n=34), the anti-IFI16 IgG levels at this time point were increased in 25 of them (74%) (Fig. 4D, right panel), and in the remaining 9 patients levels either decreased or remained unchanged compared with IFX1 (Fig. 4D,

left panel). Nineteen of the patients showing an increase in anti-IFI16 antibodies remained in clinical remission until the end of the observation period (between IFX5 and IFX13; mean observation period length: 30 weeks); 1 patient relapsed at IFX5 and did not show any further response to therapy; and 4 patients had a relapse at IFX4 or 5 before re-establishing clinical remission. By contrast, of the 8 patients who did not respond to induction therapy (n=8), none of them displayed any significant changes in anti-IFI16 IgG subtype antibody levels (data not shown).

DISCUSSION

In this prospective study we report for the first time the presence of significantly higher anti-IFI16 IgG titres in both CD and UC patients compared with HC. In addition, ELISA for anti-IFI16 IgA detection revealed slightly higher titres in CD compared with both UC and HC. In the same cohort, we also evaluated anti-GP2 antibody levels and confirmed the findings previously reported.[35-41] Anti-GP2 IgG subtype titres were significantly increased in CD as was the IgA subtype although to a lesser extent compared with UC and HC, with a statistically significant correlation between IgG and IgA subtype expression levels.

The presence of anti-IFI16 IgG antibodies in sera has previously been reported by our group and by others to be associated with a series of autoimmune diseases, while this is the first study also describing anti-IFI16 IgA distribution in parallel.[30-32, 34] In some of these previous studies, increased aberrant IFI16 expression has been demonstrated in target tissues, including the skin in SLE and the salivary glands in SjS.[33, 35] Consistent with these findings and a previous report by Vanhove et al., we were able to demonstrate aberrant epithelial IFI16 expression in inflamed colon mucosa from both CD and UC patients by immunohistochemistry.[12] Considering that: i) gut epithelial cells do not express IFI16 in the normal setting; ii) IFI16 is a nuclear DNA sensor for pathogen exogenous DNA; and iii) IBD likely results from an aberrant immune response against micro-organisms, bacteria or viruses that are able to invade cells of the gut mucosa and release or produce exogenous

dsDNA, it is not surprising that pattern recognition receptors, such as IFI16, are aberrantly upregulated in these patients and may trigger the production of specific autoantibodies.[12-19] Moreover, considering that the patients in the present study also developed mucosal IgA subtype autoantibodies, the body of evidence suggesting that IFI16 *de novo* overexpression in gut epithelial cells from IBD patients leads to a breakdown of tolerance against the IFI16 protein with the development of specific autoantibodies becomes ever the more consistent.

The logistic regression analysis for associations between the four tested antibodies and the demographical-clinical parameters of the study patients revealed some interesting findings, especially in CD, including a statistically significant association between i) elevated levels of anti-IFI16 IgG subtype with ileocolonic localization (L3) and stricturing or penetrating (B2 or B3) disease; ii) anti-IFI16 IgA with colonic localization (L2) and the presence of perianal manifestation; iii) anti-GP2 IgG with ileocolonic localization (L3) and non-stricturing and non-penetrating behaviour (B1); and iv) anti-GP2 IgA with ileal and ileocolonic localization. In UC, the only statistically significant association was between anti-IFI16 IgG and extensive colitis (E3).

Anti-GP2 IgG and IgA were more likely to occur in the same CD patients as demonstrated by a statistically significant linear regression curve, while no other concomitant occurrence was identified for the other markers. The only interesting observation was the presence of anti-IFI16 IgA antibodies in 5 CD patients negative for anti-GP2 antibodies of either subtype. This finding, together with the association of IFI16 IgA antibodies with colonic localization, may help identify CD patients that are nevertheless negative for anti-GP2 antibodies. The characteristics of these patients therefore merits further investigations.

However, the observation of most clinical interest was found by correlating the levels of the four markers with the outcome of IFX therapy: patients with low levels of IgG antibodies against IFI16 before therapy displayed a significantly higher probability of clinical response or remission after therapy. Furthermore, this was a longitudinal study in which the levels of anti-IFI16 were repeatedly measured over time; and at the IFX3 time point in particular (just before the third

infusion of the induction therapy) significant modifications in the levels of the anti-IFI16 IgG subtype were observed, especially in the CD population. An increase at this time point significantly correlated with clinical remission or response, and the majority of the patients with a documented rise of anti-IFI16 IgG remained in clinical remission until the end of the observation period. Of note, the patients who did not respond to induction therapy did not display any significant changes in anti-IFI16 IgG subtype antibody levels. The antibody levels for anti-IFI16 IgG and IgA subtypes were monitored for up to 86 weeks of therapy without showing any substantial modification in their status compared to those observed after the induction therapy (IFX3).

In addition to anti-IFI16 antibodies, IFI16 protein was also recently found to be present in sera from patients with various autoimmune diseases.[29, 31, 35] The highest serum levels of IFI16 protein were found in patients with rheumatoid arthritis.[29, 31] Despite this evidence, we failed to detect any circulating IFI16 protein in IBD patients using the same ELISA sandwich used in our previous reports (data not shown). IFI16 protein might go undetected if it is being sequestrated in the gut mucosa and lumen or, more likely, if it is being masked by other serum components present in this clinical setting (preliminary results by V.C., M.G. and M.D.A., ongoing research).

Overall, the findings presented in this study deserve more investigation in order to verify the possibility of exploiting a patient's reactivity against the IFI16 autoantigen as a predictive marker of response to IFX therapy. Anti-IFI16 IgG titres may be of great clinical relevance and provide guidance when tailoring therapeutic choices to individual patients.

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REFERENCES

1. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol.* 2010;28:573-621.
2. Laass MW, Roggenbuck D, Conrad K. Diagnosis and classification of Crohn's disease. *Autoimmun Rev.* 2014;13:467-71.
3. Conrad K, Roggenbuck D, Laass MW. Diagnosis and classification of ulcerative colitis. *Autoimmun Rev* 2014;13:463-6.
4. Baumgart DC, Sandborn WJ. Crohn's disease. *Lancet.* 2012;380:1590-1605.
5. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomics sequencing. *Nature.* 2010;464:59-65.
6. Alexander KL, Targan SR, Elson CO 3rd. Microbiota activation and regulation of innate and adaptive immunity. *Immunol Rev.* 2014:260:206-220.
7. Pastorelli L, De Salvo C, Mercado JR, et al. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Front Immunol.* 2013;4:280.
8. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature.* 2011;474:307-317.
9. Papp M, Lakatos PL. Serological studies in inflammatory bowel disease: how important are they? *Curr Opin Gastroenterol.* 2014;30:359-364.
10. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol.* 2010;10:131-144.
11. Geremia A, Biancheri P, Allan P, et al. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun Rev.* 2015;14:231-245.
12. Vanhove W, Peeters PM, Staelens D, et al. Strong upregulation of AIM2 and IFI16 inflammasomes in the mucosa of patients with active inflammatory bowel disease. *Inflamm Bowel Dis.* 2015;21:2673-2682.

13. Aguilera M, Darby T, Melgar S. The complex role of inflammasomes in the pathogenesis of Inflammatory Bowel Diseases - lessons learned from experimental models. *Cytokine Growth Factor Rev.* 2014;25:715–730.
14. Rossi O, van Baarlen P, Wells JM. Host-recognition of pathogens and commensals in the mammalian intestine. *Curr Top Microbiol Immunol.* 2013;358:291–321.
15. McGuckin MA, Eri R, Simms LA, et al. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis.* 2009;15:100-113.
16. Dempsey A, Bowie AG. Innate immune recognition of DNA: A recent history. *Virology.* 2015;479-480:146-152.
17. Alnemri ES. Sensing cytoplasmic danger signals by the inflammasomes. *J Clin Immunol*. 2010;30:512-519.
18. Brennan K, Bowie AG. Activation of host pattern recognition receptors by viruses. *Curr Opin Microbiol.* 2010;13:503-507.
19. Hu S, Peng L, Kwak YT, et al. The DNA sensor AIM2 maintains intestinal homeostasis via regulation of epithelial antimicrobial host defence. *Cell Rep* 2015;13:1922-1936.
20. Vijay-Kumar M, Sanders CJ, Taylor RT, et al. Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest.* 2007;117:3909-3921.
21. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, et al. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell.* 2004;118:229-241.
22. Maeda S, Hsu LC, Liu H, et al. Nod 2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science.* 2005;307:734-738.
23. Asquith M, Powrie F. An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer. *J Exp Med.* 2010;207:1573-1577.
24. Gariglio M, Mondini M, De Andrea M, et al. The multifaceted interferon–inducible p200 family proteins: from cell biology to human pathology. *J Interferon Cytokine Res.* 2011;31:159-172.

25. Unterholzner L, Keating SE, Baran M, et al. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol.* 2010;11:997-1004.

26. Dell'Oste V, Gatti D, Gugliesi F, et al. Innate nuclear sensor IFI16 translocates into the cytoplasm during the early stage of in vitro human cytomegalovirus infection and is entrapped in the egressing virions during the late stage. *J Virol.* 2014;88:6970-6982.

27. Schattgen SA, Fitzgerald KA. The PYHIN protein family as mediators of host defences. *Immuol Rev.* 2011;243:109-118.

28. Gariano GR, Dell'Oste V, Bronzini M, et al. The intracellular DNA sensor IFI16 gene acts as restriction factor for human cytomegalovirus replication. *PLoS Pathog.* 2012;8:e1002498.

29. Gugliesi F, Bawadekar M, De Andrea M, et al. Nuclear DNA sensor IFI16 as circulating protein in autoimmune diseases is a signal of damage that impairs endothelial cells through high-affinity membrane binding. *PLoS One.* 2013;8:e63045.

30. Mondini M, Vidali M, De Andrea M, et al. A novel autoantigen to differentiate limited cutaneous systemic sclerosis from diffuse cutaneous systemic sclerosis: the interferon-inducible gene IFI16. *Arthritis Rheum.* 2006;54:3939-3944.

31. Alunno A, Caneparo V, Bistoni O, et al. The circulating interferon-inducible protein IFI16 correlates with clinical and serological features in rheumatoid arthritis. *Arthritis Care Res.* 2016;68:440-445.

32. Costa S, Mondini M, Caneparo V, et al. Detection of anti-IFI16 antibodies by ELISA: clinical and serological associations in systemic sclerosis. *Rheumatology (Oxford).* 2011;50:674-681.

33. Caneparo V, Cena T, De Andrea M, et al. Anti-IFI16 antibodies and their relation to disease characteristics in systemic lupus erythematosus. *Lupus.* 2013;22:607-613.

34. McMahan ZH, Shah AA, Vaidya D, et al. Anti-IFI16 antibodies in scleroderma are associated with digital gangrene. *Arthritis Rheumatol.* 2015; doi: 10.1002/art.39558.

35. Alunno A, Caneparo V, Carubbi F, et al. Interferon gamma-inducible protein 16 in primary Sjögren's syndrome: a novel player in disease pathogenesis? *Arthritis Res Ther.* 2015;17:208.

36. Roggenbuck D, Reinhold D, Schierack P, et al. Crohn's disease specific pancreatic antibodies: clinical and pathophysiological challenges. *Clin Chem Lab Med.* 2014;52:483-494.
37. Roggenbuck D, Reinhold D, Wex T, et al. Autoantibodies to GP2, the major zymogen granule membrane glycoprotein, are new markers in Crohn's disease. *Clin Chim Acta.* 2011;412:718-724.
38. Roggenbuck D, Röber N, Bogdanos DP, et al. Autoreactivity to isoforms of glycoprotein 2 in inflammatory bowel disease. *Clin Chim Acta.* 2015;442:82-83.
39. Bogdanos DP, Roggenbuck D, Reinhold D, et al. Pancreatic-specific autoantibodies to glycoprotein 2 mirror disease location and behaviour in younger patients with Crohn's disease. *BMC Gastroenterol.* 2012;12:102.
40. Papp M, Sipeki N, Tornai T, et al. Rediscovery of the anti-pancreatic antibodies and evaluations of their prognostic value in a prospective clinical cohort of Crohn's patients: the importance of specific target antigen (GP2 and CUZD1). *J Crohns Colitis.* 2015;9:659-668.
41. Bogdanos DP, Rigopoulou EI, Smyk DS, et al. Diagnostic value, clinical utility and pathogenic significance of reactivity to the molecular targets of Crohn's disease specific-pancreatic autoantibodies. *Autoimmun Rev.* 2011;11:143-148.
42. Bonneau J, Dumestre-Perard C, Rinaudo-Gaujous M, et al. Systematic review: new serological markers (anti-glycan, anti-GP2, anti-GM-CSF Ab) in the prediction of IBD patient outcomes. *Autoimmune Rev.* 2015;14:231-245.
43. Hase K, Kawano K, Nochi T, et al. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. *Nature.* 2009:462:226-230.
44. Ohno H, Hase K. Glycoprotein 2 (GP2): grabbing the FimH bacteria into M cells for mucosal immunity. *Gut Microbes.* 2010;1:407-410.
45. Dignass A, Van Assche G, Lindsay JO, et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. *J Crohns Colitis.* 2010;4:28-62.

46. Dignass A, Eliakim R, Magro F, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *J Crohn Colitis.* 2012;6:965-990.
47. Dignass A, Eliakim R, Magro F, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 2: definitions and diagnosis. *J Crohn Colitis.* 2012;6:991-1030.
48. Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet.* 1980;1:514.
49. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med.* 1987;317:1625-1629.
50. Gariglio M, Azzimonti B, Pagano M, et al. Immunohistochemical expression analysis of the human interferon-inducible gene IFI16, a member of the HIN200 family, not restricted to hematopoietic cells. *J Interferon Cytokine Res.* 2002;22:815-821.

FIGURE LEGENDS

Figure 1. IFI16 expression in colon mucosa. Immunohistochemistry for the detection of IFI16 protein in tissue sections from colonic samples from control individuals (left column, panels a, d and g) and patients with active CD (central column, panels b, e and h) or UC (right column, panels c, f and i). At least 5 different cases for the different conditions were analyzed, and one representative for each is displayed. Black arrows indicate an IFI16-positive nucleus on an inflammatory cell in the lamina propria (panel g) and IFI16-positive epithelial cells (panels h and i). The dotted area in panel d highlights a small vessel with IFI16 positive endothelial cells (higher magnification in panel g, inset). The arrowhead in panel h indicates an intraepithelial inflammatory cell. Scale bars: 50 μm (a-f), 25μm (g-i).

Figure 2. Serum IgG and IgA levels specific for IFI16 and GP2 antigens in IBD patients and healthy controls. Serum IgG and IgA specific for IFI16 (A) or GP2 (B) were quantified by ELISA in healthy controls (HC) and patients suffering from CD or UC. Each dot represents the autoantibody level for each subject sample expressed in arbitrary units on a linear scale. The horizontal bars in each group represent the median values. Values over the dotted line indicate the percentage of subjects with antibody titres above the cut-off value (113 U/ml for anti-IFI16 IgG and 9.6 U/ml for anti-IFI16 IgA, calculated as the 95^{th} percentile of the control population; 15 U/ml for anti-GP2 IgG and 10 U/ml for anti-GP2 IgA, as determined by the manufacturer). Statistical significance: ***$p<0.0001$, **$p=0.001$ (Mann Whitney tests).

Figure 3. Venn diagram depicting overlap in the presence of anti-IFI16 and anti-GP2 autoantibodies (IgG and IgA subtypes) in CD patients. Numbers indicate the total number of CD patients presenting each combination of autoantibody. Of the 48 patients in the cohort, 20 were negative for both subtypes of anti-IFI16 and anti-GP2 antibodies.

Figure 4. Changes in serum IgG and IgA levels specific for IFI16 and GP2 antigens in CD patients before and after infliximab (IFX). A: Overview of the study protocol. B and C: Serum IgG and IgA specific for IFI16 (B) or GP2 (C) were quantified by ELISA in samples taken at the time of the first IFX infusion (IFX1) and at IFX3. Each dot represents the autoantibody level for each subject sample expressed in arbitrary units on a linear scale. The horizontal bars in each group represent the median values. Values over the dotted line indicate the percentage of subjects with antibody titres above the cut-off value (113 U/ml for anti-IFI16 IgG and 9.6 U/ml for anti-IFI16 IgA, calculated as the 95^{th} percentile of the control population; 15 U/ml for anti-GP2 IgG and 10 U/ml for anti-GP2 IgA, as determined by the manufacturer). Statistical significance: ***$p<0.0001$, **$p<0.001$, Wilcoxon signed-rank test. D: Changes in serum anti-IFI16 IgG levels between the IFX1 *vs*. IFX3 time points in CD patients (n=34) showing clinical response or remission at IFX4 Nine CD patients showed no increase in anti-IFI16 antibody titres at IFX3 (left panel) compared with 25 patients who did (right panel). Each dot represents the autoantibody level for each subject sample expressed in arbitrary units on a linear scale.