Heterogeneity of subordination of the IL-18/IFN- γ axis to caspase-1 among patients with Crohn's disease

Anne Jarry¹, Céline Bossard^{1,2}, Laure Droy-Dupré^{1,2}, Christelle Volteau³, Arnaud Bourreille^{4,5}, Guillaume Meurette⁶, Jean-François Mosnier^{1,2} and Christian L Laboisse^{1,2}

In Crohn's disease (CD), hierarchical architecture of the inflammatory network, including subordination of IL-18, an IFN- γ -inducing cytokine, to the inflammasome, have remained undeciphered. Heterogeneity among patients of such a subordination cannot be evaluated by animal models, monofactorial in their etiology and homogenous in disease progression. To address these issues, we set up an *ex vivo* model of inflamed mucosa explant cultures from patients with active long-standing CD. Th1 cytokine production, especially IFN- γ and IL-18, was assessed in relation with inflammation intensity. Subordination of the Th1 response to caspase-1, effector of the inflammasome, was determined in explant cultures subjected to pharmacological inhibition of caspase-1 by YVAD. We showed a correlation between secreted IFN- γ /IL-18 levels, and caspase-1 activation, with inflammation intensity of intestinal CD mucosa explants. Inhibition of caspase-1 activation using the specific inhibitor YVAD identified a homogenous non responder group featuring a caspase-1-independent IL-18/IFN- γ response, and a heterogenous responder group, in which both IL-18 and IFN- γ responses were caspase-1-dependent, with a 40–70% range of inhibition by YVAD. These findings bring out the concept of heterogeneity of subordination of the Th1 response to inflammasome activation among CD patients. This *ex vivo* model should have therapeutic relevance in allowing to determine eligibility of CD patients for new targeted therapies.

Laboratory Investigation (2015) **95**, 1207–1217; doi:10.1038/labinvest.2015.89; published online 13 July 2015

Initiator events thought to be involved in the pathogenesis of Crohn's disease (CD), a complex inflammatory bowel disease (IBD),¹ all converge to a dysregulation of the intestinal mucosa immune homeostasis. They include a downregulation of IL-10 and/or transforming growth factor- β (TGF- β) signaling—cytokines which have pivotal immunoregulatory functions in the human intestinal mucosa^{2–4} which has been reported in subgroups of CD patients.^{5–8} It is likely that these initiator events are no more drivers in a sustained, chronic intestinal inflammation like CD. Indeed, the clinical studies published indicate no relevant advantage of IL-10 therapy in CD.^{9–11} So, the subordination to initiator events seems to progressively change over time in the perpetuation of the inflammation in CD, and thus biological therapies of long-standing CD cannot be predicted on the basis of the delineation of initiator events. In fact, when considering the biological therapies of long-standing CD, the issue at stake is how to decipher the driving signaling pathways. Interestingly, anti-tumor necrosis factor- α (TNF- α) therapy, which has

significant benefit for CD patients in terms of clinical remission^{12–14} is an extension of clinical studies in rheumatoid arthritis and has not been identified by tracking a scientific concept.¹⁵

Numerous murine models of chronic intestinal inflammation close to human IBD have been established in order to study IBD pathogenesis, and much hope has been placed in predicting in these models the efficacy of new drugs in human IBD. However, very few of these murine models has proven useful to select a relevant biological therapy amenable to the clinical practice. For example, anti-TNF- α treatment can ameliorate or aggravate inflammation, depending on the mouse model used.¹⁶

Therefore, it is important to setup a preclinical model able to decipher the driving signaling pathways that maintain a sustained inflammation in CD. Our reasoning was that *ex vivo* explant cultures of inflamed mucosa from full blown CD patients can be relevant to the *in vivo* situation, and that their pharmacological manipulation can help decipher the

¹Université de Nantes, EA 4273 Biometadys, Faculté de Médecine, Nantes, France; ²CHU Nantes, Service d'Anatomie et Cytologie Pathologiques, Nantes, France; ³CHU Nantes, Plateforme de Biométrie, Nantes, France; ⁴CHU Nantes, Institut des Maladies de l'Appareil Digestif (IMAD) et Service d'Hépatogastroentérologie, Nantes, France; ⁵UMR INSERM 913, Faculté de Médecine, Nantes, France and ⁶CHU Nantes, Clinique de Chirurgie Digestive et Endocrinienne, Nantes, France

Correspondence: Dr A Jarry, PhD, Université de Nantes, EA4273 Biometadys, Faculté de Médecine, 1 Rue Gaston Veil, Nantes 44035, Cedex 1, France.

E-mail: anne.jarry@univ-nantes.fr

Received 14 January 2015; revised 26 May 2015; accepted 8 June 2015

subordination of the elements of the complex cytokine network to key molecular platforms, that can be potential targets for biotherapies. In this work, we make the hypothesis of a maintenance of the maturation pathway of proinflammatory cytokines in tissues of patients with active long-standing CD, despite the modifications resulting from disease progression and treatments. As a proof of concept, we chose to examine the maintenance of the inflammasome pathway (caspase-1–IL-18–IFN- γ axis) in full blown CD. The inflammasome is a multiprotein complex that includes the effector protein caspase-1, which is the main processing enzyme for maturation of IL-1 β and IL-18.¹⁷ IL-18 can in turn induce the production and release of IFN- γ , the prototypical Th1 cytokine.¹⁸ The human normal intestinal mucosa is a reservoir of procaspase-1 and pro-IL-18, present in epithelial cells and in some lamina propria macrophages,^{19,20} that can be rapidly activated under various stress conditions and can initiate a Th1 response.⁴ However, in a sustained chronic inflammation such as CD, the status of caspase-1 and the subordination of the Th1 response to caspase-1 activation are so far unknown.

In this work, we examined in the *ex vivo* explant culture model of inflamed mucosa from CD patients: (i) the status of the caspase-1/IL-18/IFN- γ axis in relation with the intensity of histological lesions, and (ii) the subordination of the IL-18 and IFN- γ responses to caspase-1, using pharmacological inhibition and a multiparametric approach. We show here that the activation of caspase-1, as well as the IL-18 and IFN- γ responses are related to the intensity of the inflammatory lesions in active long-standing CD. Interestingly, we also show a heterogeneity in the subordination of the IL-18/IFN- γ response to caspase-1 activation in CD patients.

MATERIALS AND METHODS

Patients

Crohn's disease patients

Intestinal samples (ileum from macroscopically inflamed areas and matched adjacent colon when possible) were obtained from 28 patients undergoing ileocelectomy or colectomy for CD between 2011 and 2013 (University Hospital of Nantes). The main clinical features of the CD patients and their treatment before surgery are mentioned in Table 1.

Normal controls

Intestinal samples were also collected from macroscopically and microscopically unaffected colon of eight patients undergoing surgery for colorectal cancer, taken at least at 10 cm downstream the tumor.

All tissue fragments were processed according to the French guidelines for research on human tissues. Informed patient consent was obtained according to the French bioethics law.

Explant Cultures

The human normal colonic mucosa from 'normal controls' ($n = 8$) was carefully stripped from the underlying compartment as previously described.^{3,4} Regarding inflamed tissue

from CD patients ($n = 28$), the muscle layers and most of the submucosa were removed. Fragments of about 40 mg each were cut, pinned in Sylgard-coated Petri dishes, and maintained for 24 h in culture in RPMI/bovine serum albumin (BSA)/antibiotics/fungizone, as previously described.³ The explants were maintained at 37 °C in a 95% oxygen, 5% carbon dioxide humid atmosphere on a rocking platform at low speed for 24 h. At the end of the 24 h culture, the supernatants were centrifuged and aliquots were stored at –80 °C for further analysis. Each cultured explant was cut into two fragments: one was used for morphological/immunohistochemical analyses after formalin fixation and paraffin embedding (see below), the other was stored at –80 °C for further analysis (eg caspase-1 activity). Inflamed mucosa from 21 CD patients was cultured in the absence or in the presence of the caspase-1 inhibitor YVAD-FMK (2 μ M; Biovision, Clinisciences, Montrouge, France). For each patient, at least four mucosa explants were put in culture for each experimental condition. After the 24-h incubation, culture medium was collected, centrifuged and stored at –80 °C until use. Tissue fragments were stored at –80 °C, and processed in several cases for determination of caspase-1 activity. Before culture, four microdissected explants were fixed in formalin, embedded in paraffin, and subjected to histological analysis (Hematoxylin-eosin (HE) standard staining) to assess inflammation as follows: no inflammation; weak inflammation (a few neutrophils and mild inflammatory infiltrate as well as mild abnormal villus/crypt architectural abnormalities); or strong inflammation (ulcerations, strong inflammatory infiltrate with many neutrophils, loss of mucosal architecture). HE staining was also used to assess the overall morphology before culture (T0) and after a 24-h culture. In addition, immunohistochemistry using a M30 antibody (Roche Diagnostics, 1:100), specific for apoptotic epithelial cells,^{21,22} and an antibody directed to active caspase-3 (Cell Signaling, 1:800), which labels all apoptotic mucosa cells (epithelial and immune cells) was performed both at T0 and after the 24-h culture. As shown in Supplementary Figure S1, the 24-h culture does not alter the overall architecture, as well as the pattern of epithelial or immune cells apoptosis, compared with T0.

Cytokine Determination Assay

The following cytokines were determined by enzyme-linked immunosorbent assay (ELISA) in samples of culture medium collected at time point 24 h, according to the manufacturers' protocols: IL-18 (Bender MedSystems; eBiosciences, Paris, France), IFN- γ (Eurobio, Courtaboeuf, France), IL-17 A (PeproTech, Neuilly, France). Results are expressed as pg/ml.

Caspase-1 Activity Assay

Inflamed mucosa explants from CD patients before culture were lysed for 1 h on ice, centrifuged at 12 000 g for 20 min. Caspase-1 activity was assessed in the lysates using the Caspase-1 Colorimetric Assay Kit (Biovision, Clinisciences,

Table 1 Main clinical features of patients with Crohn's disease (n = 28)

Case number	Sex/age at surgery	Duration of disease	Treatment at the time of surgery	Cumulative phenotype ^a	Explant cultures ^b	Microscopic inflammation
1	W, 39	15 years	Anti-TNF- α antibody	L2 colonic B2 stricturing Perianal disease	Colon	Strong
2	W, 49	24 years	Anti-TNF- α antibody Azathioprine	L3 ileocolonic B3 penetrating	Colon	weak
3	M, 23	7 years	Anti-TNF- α antibody Azathioprine	L3 ileocolonic B3 penetrating Perianal disease	Colon	Strong
4	M, 56	19 years	Anti-TNF- α antibody	L3 ileocolonic B2 stricturing	Colon	Strong
5	W, 20	16 months	Azathioprine	L3 ileocolonic B3 penetrating	Ileum	Strong
6	M, 19	1 years	Anti-TNF- α antibody	L3 ileocolonic B3 penetrating	Ileum	Strong
7	W, 32	17 years	Azathioprine	L1 ileal B2 stricturing	Ileum	Strong
8	W, 33	3 years	Anti-TNF- α Azathioprine	L1 ileal B3 penetrating	Ileum	weak
9	W, 50	21 years	Anti-TNF- α antibody	L3 ileocolonic B2 stricturing	Colon	weak
10	W, 19	11 years	Anti-TNF- α antibody	L3 ileocolonic B3 penetrating	Ileum Colon	Strong weak
11	W, 29	7 years	Azathioprine	L3 ileocolonic B2 stricturing	Ileum	weak
12	W, 21	2 years	Anti-TNF- α antibody Azathioprine	L3 ileocolonic B3 penetrating	Ileum	Strong
13	M, 71	7 years	Anti-TNF- α antibody	L1 ileal B2 stricturing Perianal disease	Ileum	Strong
14	W, 21	16 years	Anti-TNF- α antibody	L3 ileocolonic B2 stricturing	Ileum Colon	Strong weak
15	M, 25	13 years	Antibiotics	L3 ileocolonic B3 penetrating perianal disease	Ileum	Strong
16	M, 36	11 years	Methotrexate	L1 ileal B3 penetrating	Ileum Colon	Strong weak
17	W, 28	11 years	Azathioprine	L3 ileocolonic B3 penetrating	Ileum Colon	Strong weak
18	M, 20	7 years	Anti-TNF- α antibody	L2 colonic B2 stricturing perianal disease	Colon	Strong
19	W, 45	20 years	Anti-TNF- α antibody Azathioprine	L3 ileocolonic B3 penetrating	Ileum	weak
20	W, 37	13 years	Topical steroid	L1 ileal B2 stricturing	Ileum Colon	Strong 0
21	W, 56	11 years	Topical steroid	L3 ileocolonic B2 stricturing	Ileum	Strong
22	W, 31	3 years	Anti-TNF- α antibody	L3 ileocolonic B1 non-stricturing non-penetrating	Ileum Colon	Strong weak

Table 1 Continued

Case number	Sex/age at surgery	Duration of disease	Treatment at the time of surgery	Cumulative phenotype ^a	Explant cultures ^b	Microscopic inflammation
23	W, 42	3 years	None	L3 ileocolonic B3 penetrating	Ileum	Strong
24	W, 56	24 years	Azathioprine	L3 ileocolonic B2 stricturing	Ileum	Strong
25	M, 34	21 years	Anti-TNF- α antibody	L3 ileocolonic B1 non-stricturing non-penetrating	Ileum	Strong
26	M, 20	2 years	Antibiotics	L1 ileal L4 upper gastrointestinal disease B3 penetrating	Ileum	Strong
27	W, 25	6 years	Anti-TNF- α antibody	L1 ileal B2 stricturing	Ileum Colon	Strong 0
28	M, 35	6 years	Anti-TNF- α antibody	L1 ileal B2 stricturing	Ileum	Strong

M: man; W: woman; 0: noninflamed. ^aCumulative phenotype according to the Montreal classification for Crohn's disease.³⁴ ^bExplant cultures from patients 8 to 28 ($n=21$) were treated or not with the caspase-1 inhibitor YVAD.

Nanterre, France) according to the manufacturer's instructions. Briefly, 200 μ g total protein (determined in the lysates by the Biorad DC protein assay kit), were incubated with the colorimetric substrate for caspase-1 (YVAD-pNA) for 2 h at 37 °C in a 96-well plate. The absorbance was measured at a wavelength of 405 nm in a spectrofluorimeter (Victor 3, Perkin Elmer).

Immunohistochemistry

Cells containing active caspase-1 were detected by immunohistochemistry on paraffin sections of CD mucosa, after antigen retrieval (citrate buffer pH 6). Sections were then stained with antibodies directed to active caspase-1 p20 antibodies (1:100; Cell Signaling, Danvers, MA, USA), or T-bet (clone 4B10, Santa Cruz, 1:200) or granzyme B (GrzB) (Novocastra, 1:50). The immunological reaction was visualized using an Envision kit (Dako, Les Ulis, France). Diaminobenzidine was used as a chromogen. Sections were counterstained with haematoxylin. The number of T-bet- and GrzB-positive cells per field was assessed by counting at least 15 consecutive fields at a $\times 400$ magnification.

Statistics

The Mann–Whitney test was used to assess differences in cytokine levels, using GraphPad Prism (GraphPad Software Inc, La Jolla, CA). Correlation between IL-18 and IFN- γ levels, analyzed using Spearman's rank correlation, as well as association between IL-18 low/high and IFN- γ low/high subgroups, tested by the Chi-square test, were performed using the SAS 9.3 software. P -values <0.05 were considered significant.

RESULTS

Validation of Intramucosal Caspase-1 Activation and IL-18 Production as Markers Related to the Inflammation Intensity in CD Explant Cultures

The *ex vivo* model of explant cultures was used to examine the spontaneous release of mature IL-18 by the inflamed mucosa of CD patients, compared with the human normal colonic mucosa of control patients. In CD, the intensity of inflammation in the small intestine or colon was determined on mucosa before culture, and evaluated as no inflammation, weak or strong inflammation, as mentioned in the Materials and Methods section. As shown in Figure 1a, the amounts of mature IL-18, assessed by ELISA in the supernatants of 24-h explant cultures were very low in the normal colonic mucosa from 'control' patients. By contrast, high to very high levels of IL-18 were released by the strongly inflamed mucosa explants of CD patients (with interindividual variations). Weakly inflamed mucosa explants secreted significantly lower levels of IL-18 compared with strongly inflamed mucosa (Figure 1a). In seven patients undergoing an ileocelectomy, paired explants of ileum and colon could be put in culture for 24 h. In all these cases, the inflammation was weak or absent in the colon and strong in the small intestine. In most cases, IL-18 levels were higher in the inflamed mucosa (ileum) than in the weakly inflamed or noninflamed mucosa (colon; Figure 1b).

When examining IL-18 levels in relation with the main clinical features of CD patients mentioned (see Materials and Methods, Table 1), we did not find association between IL-18 levels and the cumulative phenotype according to the Montreal classification, or duration of disease. Altogether, these results show that while IL-18 levels were linked to the

Figure 1 IL-18 production as a marker related to the intensity of inflammation in mucosa explant cultures from CD patients. (a) IL-18 secretion was measured by ELISA in the supernatants of 24-h explant cultures of normal mucosa as well as of weakly or strongly inflamed mucosa from CD patients. Light gray circles indicate explant cultures from the colon and black circles from the ileum. Each circle represents the mean value of four explants for a given patient. Horizontal lines represent median values of different experiments on normal mucosa from control patients and on mucosa from CD patients with either weak or strong inflammation. Statistical analysis (weakly vs strongly inflamed CD mucosa) was performed with the Mann-Whitney nonparametric test. (b) IL-18 secretion measured in paired ileum (strong inflammation) and colon (weak inflammation) explant cultures in patients undergoing an ileocelectomy. Each symbol represents the mean value of four explants. CD, Crohn's disease.

inflammation intensity, they were not related to the clinical features of CD patients.

We next assessed the status of caspase-1, known to mediate IL-18 processing, in the inflamed mucosa of CD patients, by two complementary methods: a caspase-1 activity assay, based on cleavage of a selected substrate detected by spectrophotometry, and *in situ* detection of active caspase-1 by immunohistochemistry. As shown in Figure 2 (upper panel), caspase-1 activity, determined on cell lysates of mucosa from CD patients before culture, was significantly higher in cases with severe mucosal inflammation compared with those with weak or no inflammation. *In situ* detection of active caspase-1 using anti-caspase-1 p20 antibodies showed a strong

Figure 2 Caspase-1 activation in relation with the inflammation intensity in the mucosa of CD patients. The histogram shows caspase-1 activity, detected in lysates of mucosa from CD patients before culture, using a colorimetric assay with a specific substrate (YVAD-pNA). Absorbance at 405 nm. Mean $\pm$ s.e.m. of four CD mucosae with weak inflammation (two ileum, two colon) and four CD mucosae (ileum) with strong inflammation. Statistical analysis was performed with the Mann-Whitney nonparametric test. Images show immunostaining of active caspase-1 in a weakly inflamed and in a strongly inflamed ileal mucosa from CD patients, using an antibody directed to caspase-1 p20. In the weakly inflamed mucosa (left), active caspase-1 (brown) is present in the epithelial cells of some crypts (arrows) and in some mononuclear cells of the mucosa inflammatory infiltrate (arrowheads). In the strongly inflamed mucosa (right), numerous mononuclear cells of the dense inflammatory infiltrate express active caspase-1. Nuclei are counterstained in blue with haematoxylin. The scale bar represents 100 μ m. CD, Crohn's disease.

immunostaining in a few epithelial cells in weakly and strongly inflamed CD mucosa, as well as in immune cells of the inflammatory infiltrate, more numerous in cases with strong mucosal inflammation compared with those with weak inflammation (Figure 2, lower panel).

Relation Between IL-18 and IFN- γ Responses in the Inflamed Mucosa of CD Patients

We next addressed the issue of the possible correlation between IL-18, known as an IFN- γ -inducing cytokine, and the IFN- γ response in the inflamed mucosa explant cultures from CD patients. First, IFN- γ levels released in the supernatants of 24-h explant cultures were significantly higher in the strongly inflamed mucosa than in the weakly inflamed mucosa from CD patients (mean 209 ± 34 pg/ml vs 43 ± 16 pg/ml), whereas the human normal colonic mucosa explant cultures did not produce IFN- γ (2.6 ± 0.7 pg/ml; Figure 3a). IFN- γ levels,

Figure 3 IFN- γ response and relation between IL-18 and IFN- γ responses in the inflamed mucosa of CD patients. **(a)** IFN- γ secretion was measured by ELISA in the supernatants of 24-h explant cultures of normal mucosa as well as of weakly or strongly inflamed mucosa of CD patients. Each circle represents the mean value of four explants for a given patient. Horizontal lines represent median values of the different explant cultures. Statistical analysis was performed with the Mann-Whitney nonparametric test. **(b)** The number of cells immunostained with an antibody directed to T-bet, a Th1-specific transcription factor, was evaluated on explants of normal mucosa, as well as of weakly or strongly inflamed mucosa of CD patients. In **a** and **b**, light gray circles indicate explant cultures from the colon and black circles from the ileum. **(c)** Correlation between the IL-18 and IFN- γ levels was evaluated in explant cultures of all CD patients, whatever the intensity of inflammation. Spearman coefficient: 0.32; $P = 0.068$. **(d)** Relation between IL-18 low/high levels, and IFN- γ low/high levels of CD mucosa explant cultures. These subgroups were determined with the cut-off values selected on the basis of median values of IL-18 and IFN- γ secreted by strongly inflamed CD mucosa explants (dotted lines in **(c)**). χ^2 -test: $P = 0.09$. CD, Crohn's disease.

linked in CD patients with the inflammation intensity, were not related to the clinical features of patients. Noteworthy, the number of cells immunostained with T-bet, a Th1-specific transcription factor that controls the expression of IFN γ ,²³ was significantly higher in the strongly inflamed mucosa than in the weakly inflamed mucosa (Figure 3b). Then, the correlation between IL-18 and IFN- γ levels was evaluated using the Spearman rank correlation coefficient test, taking into account first all the explant cultures from CD patients whatever the intensity of inflammation (weak or strong). No significant correlation was found between IL-18 and IFN- γ levels (although there was a tendency ($P = 0.068$); Figure 3c). Then, a cut-off value selected on the basis of median values of both IL-18 and IFN- γ secreted by strongly inflamed mucosa explants (dotted lines in Figure 3b), allowed us to determine subgroups (IL-18 low or high and IFN- γ low or high). As shown in Figure 3d, there were concurrent high IL-18 and

IFN- γ levels or low IL-18 and IFN- γ levels in 23/34 explant cultures from 28 CD patients ie, in 68% cases, and no relation in 32% cases (11/34). In this context, the determination of the levels of two cytokines released by inflamed mucosa explant cultures is insufficient to infer a hierarchical subordination between these cytokines.

Subordination of the IL-18 and IFN- γ Responses to Caspase-1 Activation in the Inflamed Mucosa of CD Patients

In order to determine whether the IL-18 and IFN- γ responses were dependent on caspase-1 activation, inflamed mucosa explant cultures from CD patients ($n = 21$ cases) were incubated or not in the presence of the specific caspase-1 inhibitor YVAD-FMK (2 μ M) for 24 h. The cytokine response was considered to be caspase-1-dependent when the level of the cytokine was significantly lower in the presence of

Figure 4 Subordination of the IL-18 and IFN γ responses to caspase-1 activation in the inflamed mucosa of CD patients. Inflamed mucosa explant cultures from CD patients ($n=21$) were treated or not with the caspase-1 inhibitor YVAD-FMK ($2\mu\text{M}$) for 24 h. IL-18 and IFN- γ levels were measured by ELISA in the supernatants. The IL-18 or IFN- γ response was considered as YVAD-dependent when the level of the cytokine was significantly lower in the presence of YVAD compared with control cultures without YVAD. The table (a) shows the number of cases with caspase-1-dependent or -independent cytokine responses. The charts in b and c illustrate the cytokine responses measured by ELISA in the supernatants of 24-h explant cultures with and without YVAD for a given patient. In the YVAD-dependent group (b), the cytokine response was significantly lower in the presence of YVAD compared with control cultures without YVAD. In the YVAD-independent group (c), YVAD did not significantly modify the cytokine response. Each symbol represents the mean value of four explants. CD, Crohn's disease.

YVAD compared with control cultures without YVAD (at least four inflamed mucosa explants per condition in a given patient). As shown in Figure 4a, the IL-18 response was dependent on caspase-1 activation in 43% cases, and the IFN- γ response was dependent of caspase-1 in 33% cases (the percent inhibition of the IL-18 and IFN- γ responses by YVAD ranged from 40 to 70%). In addition, in a given patient, when the IL-18 response was caspase-1-dependent, the IFN- γ

response was also caspase-1-dependent in 70% cases; in the other 30% cases, the IFN- γ response (caspase-1-independent) was very low. When the IL-18 response was independent of caspase-1, the IFN- γ response was also in all cases caspase-1-independent. In some of the cases in which the IL-18/IFN- γ response was independent of YVAD, the efficacy of YVAD was verified by assessing caspase-1 activity, which was lower in YVAD-treated explant cultures compared with control

Figure 5 Granzyme B (GrzB) expression in CD mucosa, in relation with the inflammation intensity and the subordination of the IL-18 response to caspase-1. The number of GrzB-positive cells was evaluated on explants of normal mucosa, and of weakly or strongly inflamed mucosa of CD patients (a), as well as on CD mucosa with a YVAD-dependent or YVAD-independent IL-18 response. Images show representative cases of GrzB immunostaining in the ileal mucosa of a patient with a YVAD-dependent IL-18 response (few GrzB-positive cells in brown, arrows) and in a patient with a YVAD-independent IL-18 response (more numerous GrzB-positive cells in brown). In a and b, light gray circles indicate explant cultures from the colon and black circles from the ileum. The scale bar on the images represents 50 μ m. CD, Crohn's disease.

explant cultures. Figure 4b and c show cases with YVAD-dependent and YVAD-independent cytokine responses, respectively.

We next examined the possible involvement of an alternate pathway, which could be responsible for the caspase-1-independent IL-18 response in Crohn's disease patients. A potential candidate could be granzyme B (GrzB), a serine

protease known to cleave IL-18 in *in vitro* assays,²⁴ and to process IL-18 independently of caspase-1 in keratinocytes.²⁵ Interestingly, the number of GrzB-positive cells was significantly higher in strongly inflamed CD mucosa compared with weakly inflamed CD mucosa (Figure 5a). We next examined the possible correlation between the number of GrzB-positive cells and the subordination of the IL-18 response to caspase-1. As shown in Figure 5b, the number of GrzB-positive cells was significantly higher in the group of patients with a YVAD-independent IL-18 response than in the group of patients with a YVAD-dependent IL-18 response.

IL-17 Response in the Inflamed Mucosa of CD Patients

As IL-17 is a known component of the immune response in CD,^{4,26} we examined the levels of IL-17A released in the supernatants of the 24-h cultures. As shown in Figure 6a, IL-17A levels, very low in normal colonic mucosa and in weakly inflamed mucosa from CD patients, were moderate to high in strongly inflamed mucosa from CD patients (mean: 152 ± 31 pg/ml). In seven patients undergoing an ileocolicectomy, paired fragments of both ileum (strong inflammation) and colon (weak or no inflammation) could be put in culture for 24 h. In cases in which IL-17 levels were high in the inflamed ileum, IL-17 levels were significantly lower in the weakly inflamed colon (Figure 6b). Noticeably, YVAD was unable to modify the IL-17 response, which served as a 'negative control' in our experiments (Figure 6c). Altogether, our study established IL-17 as an inflammatory cytokine independent from the caspase-1–IL-18–IFN- γ axis.

DISCUSSION

Several studies, based on cultures of endoscopic biopsies or on isolated lamina propria mononuclear cells from CD patients, described increased levels of various proinflammatory cytokines levels produced in active, long-standing CD, including TNF- α , IFN- γ , IL-18, IL-17, IL-12, and IL-23.^{26–29} However, it remains unknown how these cytokines are integrated in the network driving the evolution of the disease. Recently, a new cytokine taxonomy of human chronic inflammatory diseases has been proposed, based on clinical trials aimed at defining the potential driver cytokines.³⁰ However, in this hierarchical taxonomy, little is known about the cytokine framework of CD, whose main driving cytokine rests on TNF- α . In this context, we propose a new preclinical approach aimed at evaluating (i) the subordination hierarchy of the cytokine network operating in active, long-standing CD, and (ii) the potential heterogeneity of this subordination.

In this work, we first evaluated the hierarchical subordination of the IL-18–Th1 axis to caspase-1 activation in full blown CD, using *ex vivo* explant cultures of inflamed mucosa subjected or not to pharmacological inhibition of caspase-1. Our rationale for focusing on the caspase-1/IL-18 axis arose out of our previous studies showing that in the human normal intestinal mucosa, activation of the epithelial and macrophage inflammasome is the key to the initiation of

Figure 6 IL-17 response in the inflamed mucosa of CD patients. (a) IL-17A secretion was measured by ELISA in the supernatants of 24-h explant cultures of normal mucosa, as well as of weakly or strongly inflamed mucosa of CD patients. Light gray circles indicate explant cultures from the colon and black circles from the ileum. Each circle represents the mean value of four explants for a given patient. Horizontal lines represent median values. Statistical analysis was performed with the Mann–Whitney nonparametric test. (b) IL-17 secretion measured in 24-h explant cultures of paired ileum (strong inflammation) and colon (weak inflammation), in patients undergoing an ileocelectomy. (c) IL-17A secretion was measured by ELISA in the supernatants of 24-h explant cultures treated with or without YVAD. The IL-17 response was not significantly modified by YVAD. In **b** and **c**, each symbol represents the mean value of four explants. CD, Crohn's disease.

a Th1 inflammatory response.⁴ Up to now, the role of caspase-1 in a sustained intestinal inflammation has been studied mainly in mouse models with a genetic deficiency in a component of the inflammasome pathway including caspase-1, in association with a chemical inflammatory stress (DSS or TNBS).³¹ However, these experimental conditions are far from the human inflammatory bowel diseases. In fact, these animal models provide clues only on the caspase-1-dependent susceptibility of the mouse model to a chemically induced stress. These models do not provide information on the role of caspase-1 in CD. Interestingly, a very recent report emphasizes the important role of the inflammasome-caspase-1 pathway in driving the progression of inflammation in the IL10-deficient mouse model of enterocolitis.³² In fact, in this model, caspase-1 inhibition had a strong beneficial effect. However, the IBD mimicry of this model is very limited. First, it results from a monogenic alteration, whereas human IBD is thought to have a multifactorial origin. In addition, the choice of a unique inbred line of mice

guarantees homogeneous responses to the manipulation of caspase-1. These are severe limitations that can only miss the crucial issue of the heterogeneity of the subordination of the downstream targets of caspase-1 to caspase-1 inhibition.

In this context, the best model for studying the cytokine pathway in CD is obtained by translating the lesion itself *ex vivo*. The first step using this approach was to show a correlation between the levels of IFN- γ , IL-18, caspase-1 activation, and the intensity of the inflammatory lesions in CD. This finding suggests that the caspase-1–IL-18–IFN- γ axis is conserved in active, long-standing CD, and could have a role in the maintenance of a sustained inflammation, whatever the clinical phenotype of CD patients. However, comparison of the levels of two cytokines (IL-18 and IFN- γ) does not inform about their hierarchical order and their potential subordination to caspase-1 activation. This issue can be addressed using a pharmacological approach, ie, an inhibition of caspase-1 activation by the caspase-1 inhibitor YVAD. This approach allowed us to demonstrate an heterogeneity of the

subordination of the IL-18–IFN- γ response to caspase-1 activation in CD patients. Indeed, we define herein a population of patients with a caspase-1-dependent IL-18–IFN- γ response, and another population of patients with a caspase-1-independent IL-18–IFN- γ response. This latter population suggests the existence of alternate pathways of activation of the IL-18–IFN- γ axis. There are several candidate proteins such as proteinase-3 and the serine protease GrzB.³³ Evidence from the potential involvement of these candidate proteins is often indirect. To our knowledge, only GrzB was found to be involved in the maturational cleavage of IL-18 in *in vitro* assays.^{24,25} Interestingly, we found an increased number of GrzB-positive cells in the inflamed mucosa of CD patients, in relation with the inflammation intensity. In addition, GrzB-positive cells were significantly higher in patients with a YVAD-independent IL-18 response than in patients with a YVAD-dependent IL-18 response.

In conclusion, here we show for the first time that the human *ex vivo* model of intestinal inflamed mucosa explant cultures from CD patients is a powerful tool, not only to qualitatively and quantitatively evaluate inflammatory cytokines, but also to determine the hierarchical subordination of cytokines to a given signaling pathway. This approach is crucial for developing a hierarchical taxonomy of cytokines in CD. In addition, our study brings out the concept of the heterogeneity of the subordination of the Th1 response to inflammasome activation among patients. This concept of heterogeneity is the basis for developing targeted therapies, well known in oncology. In fact, in this field, the heterogeneity of the response to a targeted therapy requires a screening of candidate patients with available biomarkers predictive of the response. Here we show that this concept applies to CD. In fact, predicting the efficacy of a candidate biotherapy depends on the availability of a preclinical screening test. Thus, the use of mucosa explant cultures for treatment testing might be useful in this context of personalized medicine.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

ACKNOWLEDGMENTS

We thank Stéphanie Blandin and Cécile Deleine for expert technical assistance. We also thank Nicolas Leboeuf from the Photologie Department for his help. EA 4273 Biometadys is funded by the Ministère de la Recherche. AJ is the recipient of a tenure position supported by INSERM.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

- Baumgart DC, Sandborn WJ. Crohn's disease. *Lancet* 2012;380:1590–1605.
- Di Sabatino A, Pickard KM, Rampton D *et al*. Blockade of transforming growth factor beta upregulates T-box transcription factor T-bet, and increases T helper cell type 1 cytokine and matrix metalloproteinase-3 production in the human gut mucosa. *Gut* 2008;57:605–612.
- Jarry A, Bossard C, Bou-Hanna C *et al*. Mucosal IL-10 and TGF-beta play crucial roles in preventing LPS-driven, IFN-gamma-mediated epithelial damage in human colon explants. *J Clin Invest* 2008;118:1132–1142.
- Jarry A, Bossard C, Sarabayrouse G *et al*. Loss of interleukin-10 or transforming growth factor beta signaling in the human colon initiates a T-helper 1 response via distinct pathways. *Gastroenterology* 2011;141:1887–96 e1–e2.
- Monteleone G, Del Vecchio Blanco I, Monteleone I *et al*. Post-transcriptional regulation of Smad7 in the gut of patients with inflammatory bowel disease. *Gastroenterology* 2005;129:1420–1429.
- Correa I, Veny M, Esteller M *et al*. Defective IL-10 production in severe phenotypes of Crohn's disease. *J Leukoc Biol* 2009;85:896–903.
- Glocker EO, Kotlarz D, Boztug K *et al*. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 2009;361:2033–2045.
- Begue B, Verdier J, Rieux-Laucat F *et al*. Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease. *Am J Gastroenterol* 2011;106:1544–1555.
- Fedorak RN, Gangl A, Elson CO *et al*. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology* 2000;119:1473–1482.
- Herfarth H, Scholmerich J. IL-10 therapy in Crohn's disease: at the crossroads. *Treatment of Crohn's disease with the anti-inflammatory cytokine interleukin 10*. *Gut* 2002;50:146–147.
- Tilg H, van Montfrans C, van den Ende A *et al*. Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. *Gut* 2002;50:191–195.
- Sandborn WJ, Hanauer SB, Rutgeerts P *et al*. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. *Gut* 2007;56:1232–1239.
- Lemann L, Mary JY, Duclos B *et al*. Groupe d'Etude Therapeutique des Affections Inflammatoires du Tube Digestif (GETAID). Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006;130:1054–1061.
- Rutgeerts P, Vermeire S, Van Assche G. Biological therapies for inflammatory bowel diseases. *Gastroenterology* 2009;136:1182–1197.
- Feldmann M, Maini RN. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* 2001;19:163–196.
- Valatas V, Vakas M, Kolios G. The value of experimental models of colitis in predicting efficacy of biological therapies for inflammatory bowel diseases. *Am J Physiol* 2013;305:G763–G785.
- Martinson F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol* 2009;27:229–265.
- Dinarello CA. IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J Allergy Clin Immunol* 1999;103:11–24.
- Jarry A, Vallette G, Cassagnau E *et al*. Interleukin 1 and interleukin 1beta converting enzyme (caspase 1) expression in the human colonic epithelial barrier. Caspase 1 downregulation in colon cancer. *Gut* 1999;45:246–251.
- Pages F, Berger A, Henglein B *et al*. Modulation of interleukin-18 expression in human colon carcinoma: consequences for tumor immune surveillance. *Int J Cancer* 1999;84:326–330.
- Caulin C, Salvesen G, Oshima RG. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J Cell Biol* 1997;138:1379–1394.
- Leers MP, Kölgén W, Björklund V *et al*. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* 1999;187:567–572.
- Szabo SJ, Kim ST, Costa GL *et al*. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000;100:655–669.
- Omoto Y, Yamanaka K, Tokime K *et al*. Granzyme B is a novel interleukin-18 converting enzyme. *J Dermatol Sci* 2010;59:129–135.
- Akeda T, Yamanaka K, Tsuda K *et al*. CD8+ T cell granzyme B activates keratinocyte endogenous IL-18. *Arch Dermatol Res* 2014;306:125–130.
- Rovedatti L, Kudo T, Biancheri P *et al*. Differential regulation of interleukin-17 and interferon gamma production in inflammatory bowel disease. *Gut* 2009;58:1629–1636.
- Monteleone G, Trapasso F, Parrello T *et al*. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol* 1999;163:143–147.
- Pizarro TT, Michie MH, Bentz M *et al*. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J Immunol* 1999;162:6829–6835.

29. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011;140: 1756–1767.
30. Schett G, Elewaut D, McInnes IB *et al*. How cytokine networks fuel inflammation: toward a cytokine-based disease taxonomy. *Nat Med* 2013;19:822–824.
31. Zaki MH, Boyd KL, Vogel P *et al*. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* 2010;32:379–391.
32. Zhang Z, Fu S, Sun S *et al*. Inflammasome activation has an important role in the development of spontaneous colitis. *Mucosal Immunol* 2014;7:1139–1150.
33. van de Veerdonk FL, Netea MG, Dinarello CA *et al*. Inflammasome activation and IL-1 β and IL-18 processing during infection. *Trends Immunol* 2011;32:110–116.
34. Satsangi J, Silverberg MS, Vermeire S *et al*. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 2006;55:749–773.