

ORIGINAL RESEARCH

Interferon-Alpha Promotes Th1 Response and Epithelial Apoptosis via Inflammasome Activation in Human Intestinal Mucosa



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SUMMARY

Based on an ex vivo explant culture model, we decipher the immunopathologic effects of interferon alpha on the human intestinal mucosa. Interferon alpha causes a Th1 response subordinated to the activation of the inflammasome, leading to epithelial apoptosis involving altered epithelial Wnt/ β -catenin signaling.

BACKGROUND & AIMS: Several lines of investigation suggest that interferon (IFN) alpha can alter human intestinal mucosa homeostasis. These include the endogenous production of IFN alpha in celiac disease or inflammatory bowel diseases, as well as the occurrence of intestinal side effects of exogenous IFN alpha used as a therapeutic tool. Here, we present an ex vivo translational approach to investigate the effects of IFN alpha on the human normal intestinal mucosa, as well as its underlying mechanisms.

METHODS: Human normal colonic mucosa explants were cultured in the presence or absence of IFN alpha 2a. Epithelial homeostasis was assessed using the immunohistochemical marker of apoptosis M30. The Wnt inhibitor Dickkopf-Homolog-1 (DKK1) was assayed in the supernatants by enzyme-linked immunosorbent assay. Activation of the inflammasome (caspase-1/interleukin [IL]18) and of a Th1 response was determined by in situ detection of active caspase-1, as well as by measurement of mature IL18 production and the prototype Th1 cytokine IFN gamma by enzyme-linked immunosorbent assay. In addition, mechanistic studies were performed using the specific caspase-1 inhibitor Tyr-Val-Ala-Asp(OMe)-fluoromethylketone (YVAD-FMK), IL18-binding protein, neutralizing anti-IFN gamma, and anti-DKK1 antibodies.

RESULTS: IFN alpha 2a elicited a rapid (24 hours) disruption of surface and crypt colonic epithelial cells via apoptosis that was variable in intensity among the 20 individuals studied. This apoptotic effect was dependent on the initiation of an IFN gamma response elicited by resident T box expressed in T cells-positive lamina propria cells. Both apoptosis and Th1 response were subordinated to active caspase-1 and IL18 production. Finally, neutralization of IFN gamma-induced DKK1 partially protected against IFN alpha-induced epithelial apoptosis.

CONCLUSIONS: By using an ex vivo model, we show an inter-individual heterogeneity of IFN alpha effects. We show that IFN

alpha is able to disrupt both epithelial and immune homeostasis in the human intestine, by activation of an innate immunity platform, the inflammasome, which drives a Th1 response and leads to epithelial barrier disruption. (*Cell Mol Gastroenterol Hepatol* 2017;3:72–81; <http://dx.doi.org/10.1016/j.jcmgh.2016.09.007>)

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See editorial on page 4.

Type I interferons (IFNs), a family composed mainly of IFN alpha subtypes and IFN beta, are well known as innate cytokines produced early as the first line of defense against viral or bacterial infections. Type I IFNs signal via IFN alpha receptors, which are expressed ubiquitously.^{1,2} Recently, it has been reported that type I IFN can have immunomodulatory effects via direct or indirect, partially known, mechanisms.^{1,3} In addition, several reports strongly suggest that endogenously produced IFN alpha is involved in human intestinal immunopathologies such as celiac disease and inflammatory bowel diseases.^{4–6} IFN alpha, represented by IFN alpha 2a (Roferon), has been used widely in the clinic to treat cancer and viral infections. In particular, Roferon is used in hematologic malignancies and in solid tumors such as melanoma, renal cell carcinoma, hepatocellular carcinoma, as well as in viral hepatitis.^{7,8} The antitumor or antiviral effects of IFN alpha most likely are owing to activation of CD4+ and CD8+ T lymphocytes and natural killer cells by increasing their ability to kill target cells or to produce IFN gamma, in

Abbreviations used in this paper: DKK1, Dickkopf-Homolog-1; ELISA, enzyme-linked immunosorbent assay; FLICA, fluorescent-labeled inhibitor of caspases; IFN, interferon; IL, interleukin; IL18-BP, interleukin 18-binding protein; T-bet, T box expressed in T cells; Tc1, cytotoxic T cells type 1; Th, T-helper; 3D, 3-dimensional; YVAD-FMK, Tyr-Val-Ala-Asp(OMe)-fluoromethylketone.

Most current article

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mouse models.^{3,9-12} In addition, IFN alpha increases the expression of HLA class I molecules on cancer cell lines.¹³ Interestingly, IFN alpha-based therapy frequently is associated with adverse effects, including intestinal disorders (diarrhea and in rare cases pseudoinflammatory bowel disease).¹⁴ Altogether, evidence derived from intestinal immunopathologic observations and from side effects elicited by IFN alpha therapy points to the human intestinal mucosa as a target of IFN alpha by albeit still unknown mechanisms.

The current study aimed to delineate the effects of IFN alpha on the human normal intestinal mucosa and its underlying mechanisms by using a translational model. To this end, we used an ex vivo 3-dimensional (3D) model of human normal intestinal mucosa explant culture, showing all cell types and cellular interactions able to build innate and/or adaptive immune responses.^{15,16} We investigated the effects of IFN alpha 2a on the intestinal mucosa homeostasis, focusing on epithelial and immune homeostasis. We show that IFN alpha induces intestinal epithelial barrier disruption, variable in intensity among individuals, dependent on the initiation of a T-helper (Th)1 IFN gamma response elicited by resident innate/adaptive immune cells of the mucosa, involving the caspase-1/interleukin (IL)18 axis.

Materials and Methods

Human Normal Colonic Mucosa Explant Culture

Macroscopically and microscopically unaffected human normal colon was collected from 20 patients undergoing

surgery for colon cancer at the University Hospital of Nantes (10 men and 10 women; mean age, 74 y; range, 59–88 y). Patients did not undergo radiotherapy or chemotherapy and were not treated with immunosuppressive agents. The patients' main clinicopathologic features are listed in [Table 1](#). The 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.

The mucosa was stripped from the underlying submucosa and pinned onto Sylgard-coated Petri dishes (VWR, Sarasota, FL) as previously described.¹⁵ Fragments of 40 mg were maintained in culture in RPMI/Ham F12 (1:1, vol/vol)-bovine serum albumin 0.01%/antibiotics/fungizone for 24 hours, in the absence or presence of the indicated concentrations of IFN alpha 2a (PBL Assay Science, Piscataway, NJ). The explants were maintained at 37°C in a 95% O₂, 5% CO₂ humid atmosphere on a rocking platform at low speed. In each experiment, 4 explants were cultured per condition. After the 24-hour culture, the supernatants were collected, centrifuged, and aliquots were stored at -80°C for further analysis. Some explants were fixed in formalin and embedded in paraffin for histologic and immunohistochemical analyses and some explants were frozen in liquid nitrogen and stored at -80°C for further in situ assessment of caspase-1 activation (see later). Some experiments were performed for 24 hours in the presence of the caspase-1 inhibitor Tyr-Val-Ala-Asp(OMe)-fluoromethylketone (YVAD-FMK) (2 μmol/L; Biovision, Clinisciences, Montrouge, France), the IL18 antagonist IL18-binding protein (IL18-BP) (recombinant human

Table 1. Patient Clinicopathologic Features: Experiments Performed on Human Normal Colonic Mucosa Explant Cultures

Sex/age at surgery, y	Normal colon sample	Colectomy	Experiments performed on normal colonic explant cultures
M, 61	Right colon	CRC (pT3 pN0 pM0)	Control, IFN alpha (10, 100, 500 U/mL)
M, 88	Rectosigmoid	CRC (pT2 pN0 pMx)	Control, IFN alpha (10, 100, 500 U/mL)
W, 67	Right colon	CRC (pT2 pN1a pM0)	Control, IFN alpha (10, 100, 500 U/mL)
W, 89	Right colon	CRC (pT3 pN1b pMx)	Control, IFN alpha (10, 100, 500 U/mL)
W, 84	Left colon	CRC (pT4a pN2a pMx)	Control, IFN alpha (10, 100, 500 U/mL)
W, 83	Left colon	CRC (pT4b pN2b pMx)	Control, IFN alpha 500 U/mL
W, 62	Left colon	CRC (pT3 pN1b pMx)	Control, IFN alpha 500 U/mL
W, 87	Right colon	CRC (pT3 pN0 pMx)	Control, IFN alpha 500 U/mL
M, 78	Left colon	CRC (pTis pN0 pM0)	Control, IFN alpha ± YVAD
M, 83	Right colon	CRC (pT4a pN0 pMx)	IFN alpha ± YVAD or IL18-BP
W, 45	Left colon	Primary peritoneal serous adenocarcinoma	IFN alpha ± YVAD
M, 86	Left colon	CRC (pTis pNx pM0)	Control, IFN alpha 500 U/mL ± IL18-BP
M, 70	Right colon	CRC (pT1 pN0 pM0)	Control, IFN alpha ± YVAD or IL18-BP
M, 78	Left colon	CRC (pT1 pN0 pM0)	Control, IFN alpha ± anti-IFN gamma
M, 82	Right colon	CRC (pT3 pNx)	Control, IFN alpha ± IL18-BP
M, 59	Right colon	CRC (pT3 pN0 pM0)	Control, IFN alpha ± anti-IFN gamma
W, 76	Sigmoid	CRC (pT2 pN0 pMx)	IFN alpha ± anti-IFN gamma or anti-DKK1
W, 58	Left colon	CRC (pT3 pN0 pMx)	IFN alpha ± anti-DKK1
W, 84	Right colon	CRC (pT4 pN2b pMx)	IFN alpha ± anti-DKK1
W, 73	Left colon	CRC (pT1 pN0 pM0)	IFN alpha ± anti-DKK1

NOTE. pTNM stage is shown in parentheses. CRC, colorectal cancer.

IL18-BPa/Fc protein, 800 ng/mL; Bio-Techne, Lille, France), or in the presence of neutralizing anti-IFN gamma antibodies (2 µg/mL; Eurobio, Courtaboeuf, France) or anti-Dickkopf-Homolog-1 (DKK1) antibodies (5 µg/mL, Bio-technique). Table 1 lists the experiments performed using the normal colonic mucosa of each individual.

Morphology and Immunohistochemistry

Standard H&E staining was used to assess the overall morphology and viability of the explants after a 24-hour culture in the absence or presence of IFN alpha 2a on paraffin sections. To assess epithelial apoptosis, immunohistochemistry was performed using the M30 cytodeath antibody (1:200; Roche Diagnostics, Meylan, France), which recognizes a cytokeratin-18 neoepitope that becomes available at an early caspase cleavage event during apoptosis and is considered a marker of apoptosis in epithelial cells.^{17,18} An antibody directed to T bet expressed in T cells (T-bet) (clone 4B10, 1:200; Santa Cruz, Clinisciences, Nanterre, France) was used to assess Th1/cytotoxic T cells type 1 (Tc1) cells in the mucosa explant cultures. Immunohistochemistry was performed using antigen retrieval in citrate buffer with pH 6 and the BondMax automated staining system (Leica, Nanterre, France). Diaminobenzidine was used as a chromogen and a light nuclear counterstaining was performed with hematoxylin. The percentage of M30-positive apoptotic crypts was assessed by counting at least 100 well-oriented crypts in 3–4 explants after 24-hour culture at magnification of 200×. In normal untreated mucosa, only a few crypts showed numerous M30-positive apoptotic cells, usually greater than or equal to 20% total epithelial cells. Thus, colonic crypts containing at least 20% M30-positive cells along the crypt were considered positive in control and IFN alpha-treated cultures. The percentage of T-bet+ cells was assessed by counting in the lamina propria the number of T-bet+ cells and the total number of mononuclear cells in at least 5 fields at a magnification of 200×.

Cytokine Determination Assay

The following cytokines were determined by enzyme-linked immunosorbent assay (ELISA) in the same aliquots of culture medium, according to the manufacturers' instructions: IFN gamma (Eurobio, Courtaboeuf, France), IL18 and DKK1 (Bio-Techne-R&D Systems, Lille, France). Results are expressed as picograms per milliliter.

Assay of Caspase-1 Activation In Situ

Caspase-1 activation was assessed in situ on 8-µm unfixed frozen sections using the green fluorescein-fluorescent-labeled inhibitor of caspases (FLICA) caspase-1 kit (Immunohistochemistry Technologies, Bloomington, MN) according to the manufacturer's instructions. This assay uses the fluorescein inhibitor probe fluorescein-YVAD-FMK to label active caspase 1 enzyme in living cells or tissue samples. Briefly, unfixed frozen sections of cultured explants were blocked with UltraV block (ThermoScientific, Waltham, MA) for 10 minutes, incubated for 2 hours with FLICA stock

solution (1:100 dilution), and washed. Nuclei then were stained with 4',6-diamidino-2-phenylindole (1:1000; in-Vitrogen, Waltham, MA). Sections were mounted using the Prolong anti-fade medium (InVitrogen). The fluorescence was observed on a fluorescent microscope (Axiovert 200-M; Zeiss, Göttingen, Germany) equipped with an ApoTome slider. Image processing was performed using an AxioCam camera and AxioVision software (Zeiss, Goettingen, Germany).

Statistics

The Wilcoxon matched pairs test was used to assess differences in cytokine levels between control and treated explant cultures or between explants treated or not with inhibitors or neutralizing antibodies, using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). Correlation between IFN gamma and DKK1 levels and IFN gamma and T-bet+ cells, analyzed using Spearman rank correlation, as well as association between IFN gamma low/high and Tbet+ low/high subgroups, determined using the Fisher test, were performed using SAS 9.3 software (Cary, NC). *P* values less than .05 were considered significant.

Results

IFN Alpha Alters the Human Intestinal Epithelial Barrier Homeostasis

The ex vivo explant culture model of human normal colonic mucosa was used to assess the effects of IFN alpha on the overall mucosa architecture and particularly on the epithelial barrier homeostasis. To this end, explant cultures were incubated for 24 hours with various concentrations of IFN alpha and then processed for standard histologic analysis and detection by immunohistochemistry of epithelial apoptosis using the M30 antibodies. IFN alpha induced epithelial barrier disruption, both of the surface epithelium and crypt base, beginning at 100 U/mL and stronger at 500 U/mL (Figure 1A, pictures). Evaluation of the percentage of M30-positive crypts showed an apoptotic effect of IFN alpha on epithelial cells at 100 U/mL and 500 U/mL concentrations (Figure 1A). Figure 1A also shows that the apoptotic effect of IFN alpha on the epithelial barrier was heterogeneous among the 5 tested mucosae. The heterogeneity of the IFN alpha-induced apoptotic effect was confirmed further on explant cultures from 14 mucosae treated with 500 U/mL IFN alpha 2a (the 5 mucosae shown in Figure 1A and 9 other mucosae), with a number of M30-positive apoptotic cells ranging from 15% to 67% (Figure 1B). Of note, in 21% of cases (3 of 14), IFN alpha did not increase epithelial barrier apoptosis compared with control cultures.

IFN Alpha Alters the Intestinal Mucosa Immune Homeostasis: Induction of a Th1 Response

We next examined whether IFN alpha was able to modulate the intestinal mucosa immune homeostasis and particularly to induce the prototype Th1 cytokine IFN gamma. Analysis of the 24-hour explant culture supernatants by ELISA showed that baseline levels of IFN gamma (control cultures) were very low. IFN alpha elicited an IFN gamma response in 4

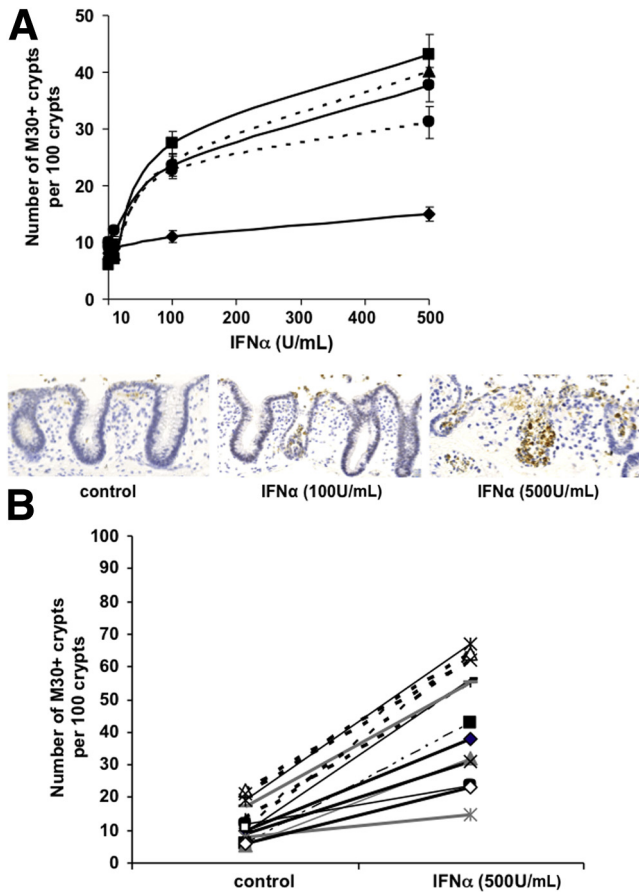


Figure 1. IFN alpha alters the human intestinal barrier homeostasis. (A) Intestinal mucosa explant cultures from 5 individuals were treated or not for 24 hours with increasing concentrations of IFN alpha 2a. The percentage of apoptotic crypts was determined after immunohistochemical staining with M30 antibodies, as mentioned in the Materials and Methods section. Representative pictures of M30 immunolabeling show that IFN alpha induces epithelial apoptosis (brown cells), both at the surface epithelium and crypt base, beginning at 100 U/mL and higher at 500 U/mL. Each symbol and bar represents the mean value and SEM of 4 explants. (B) Percentage of M30+ apoptotic crypts in paired control (untreated) cultures and in cultures treated with 500 U/mL IFN alpha in mucosae from 14 individuals. Each symbol represents the mean value of 4 explants. The variation between the 4 explants did not exceed 15%.

of the 5 mucosae tested, whose levels can vary among individuals (Figure 2A). This heterogeneity of the IFN gamma response elicited by IFN alpha (500 U/mL) was confirmed further on 14 different mucosae (the 5 mucosae shown in Figure 1A and 9 other mucosae), ranging from 20 to 560 pg/mL (Figure 2B). In 14% of cases (2 of 14), IFN alpha did not increase the IFN gamma response compared with control cultures. Interestingly, the IFN gamma levels released by IFN alpha-treated explant cultures correlated with the percentage of M30-positive apoptotic crypts (Figure 2C).

We next assessed in situ the expression of T-bet, a transcription factor known to direct Th1/Tc1 lineage commitment and to control the expression of IFN gamma.^{19,20} T-bet+ lamina propria lymphocytes, rare in control cultures, were increased in explant cultures treated with 500 U/mL IFN

alpha (Figure 2F). The number of lamina propria T-bet+ cells were counted on paraffin sections of IFN alpha-treated explants, and correlated with the levels of IFN gamma released in the supernatants. As shown in Figure 2D, there was a significant positive correlation between the percentage of lamina propria T-bet+ cells and IFN gamma levels ($P = .0006$; Spearman coefficient, 0.7). In addition, Figure 2E shows the existence of 2 subgroups of patients, with concurrent high IFN gamma and T-bet+ cells or low IFN gamma and T-bet+ cells.

We next examined whether the IFN gamma response was associated with activation of the inflammasome pathway (ie, production of mature IL18 and activation of caspase-1). Mature IL18 was assayed by ELISA in the same supernatant aliquots of the 14 mucosae showed in Figure 2B (same line code). As shown in Figure 3A, IFN alpha elicited in most individuals an increased IL18 response compared with control cultures, variable among individuals (range, 9–365 pg/mL). In 29% of cases (4 of 14), IFN alpha did not increase mature IL18 release compared with control cultures.

We next assessed the status of caspase-1, known to mediate IL18 processing, by an in situ detection of active caspase-1 using the FLICA assay (a fluorescent-labeled inhibitor of caspase-1 that irreversibly binds with activated caspase-1). In untreated control cultures, the intestinal epithelial cells did not show caspase-1 activity, apart from some extruded apoptotic cells at the surface epithelium; a few mononuclear cells were FLICA positive in the lamina propria (Figure 3B, left). Upon IFN alpha treatment, numerous FLICA-positive epithelial cells were observed in altered, apoptotic crypts (membrane and cytoplasmic staining) (Figure 3B), as well as a few FLICA-positive lamina propria mononuclear cells.

The IFN Alpha-Induced Th1 Response and Epithelial Barrier Apoptosis Are Subordinated to the Inflammasome Pathway

To test whether the Th1 (IFN gamma) response and epithelial apoptosis elicited by IFN alpha were dependent on the caspase-1/IL18 axis, IFN alpha-treated explant cultures were incubated or not in the presence of the specific caspase-1 inhibitor YVAD-FMK (2 μ mol/L) or in the presence of recombinant human IL18 binding protein (IL18-BP, 800 ng/mL), a specific antagonist of IL18. As shown in Figure 4A, both YVAD and IL18-BP significantly inhibited the IFN gamma response elicited by IFN alpha ($P = .001$ and $.008$ vs IFN alpha alone, respectively). In addition, the number of M30-positive apoptotic crypts induced by IFN alpha treatment was inhibited significantly by the inflammasome inhibitors YVAD ($P = .001$) and IL18-BP ($P = .007$) (Figure 4B, left and middle).

Finally, using the M30 apoptotic assay as an end point, we examined the sequence of causal relationships of the earlier-mentioned signaling events. As shown in Figure 4B (right), blocking IFN gamma using neutralizing antibodies in IFN alpha-treated explants led to a significant decrease in the number of M30+ apoptotic crypts ($P = .01$ vs IFN alpha alone). Knowing that the IFN gamma production elicited by IFN alpha in mucosa explant cultures is subordinated to the

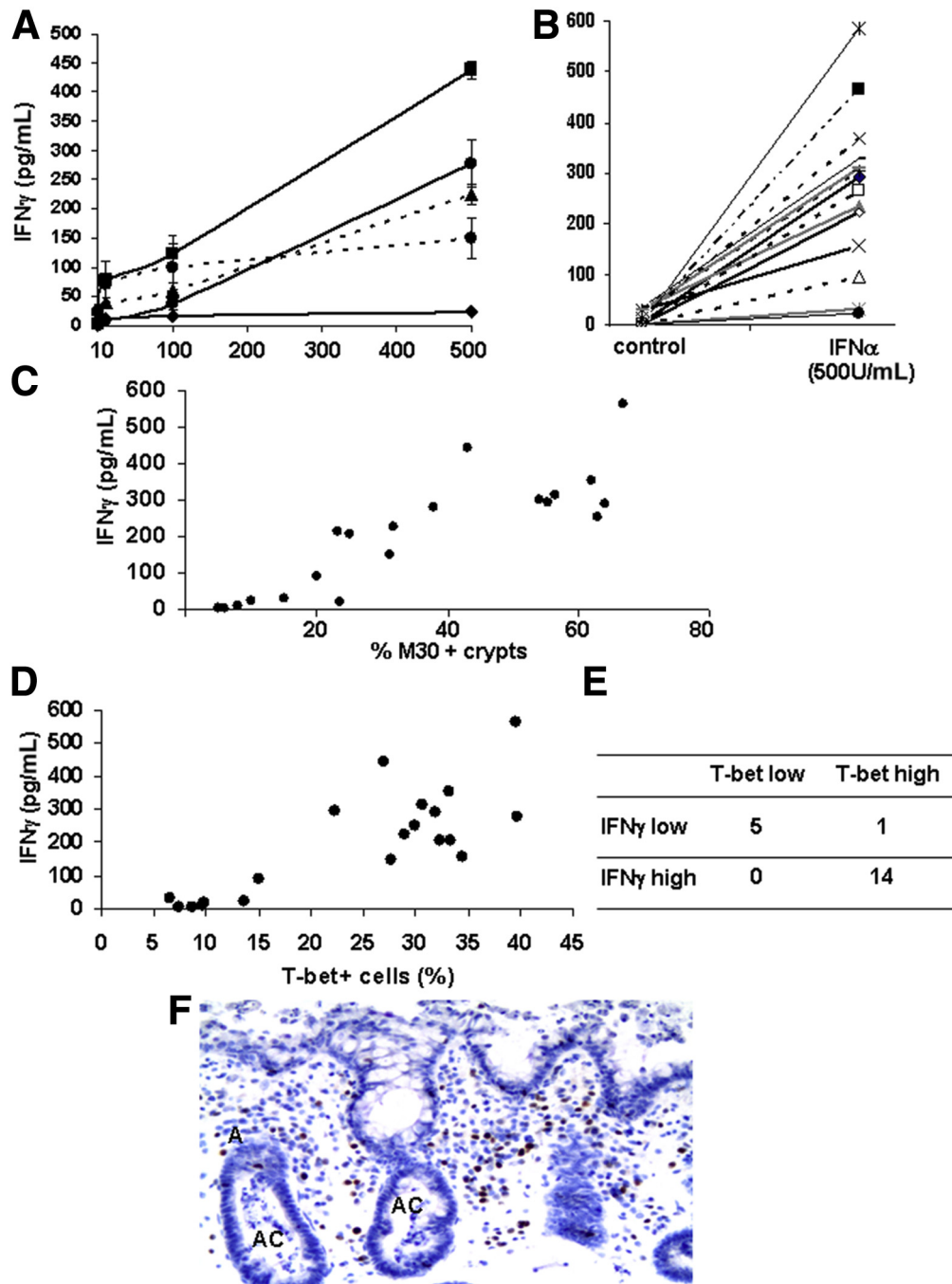


Figure 2. IFN alpha alters the human intestinal mucosa immune homeostasis and elicits a Th1 IFN gamma response. (A) Intestinal mucosa explant cultures from 5 individuals were treated or not for 24 hours with increasing concentrations of IFN alpha 2a. IFN gamma secretion was quantified by ELISA in the culture supernatants. Each *symbol* and *bar* represents the mean value and SEM of 4 explants. (B) IFN gamma was measured by ELISA in paired control and IFN alpha-treated mucosa explant cultures from 14 individuals. Each *symbol* represents the mean value of 4 explants. The variation among the 4 explants did not exceed 20%. (C) Correlation between IFN gamma levels released in the supernatants of colonic mucosa explant cultures treated with IFN alpha (500 U/mL, 24 hours) and the percentage of M30+ crypts. Spearman coefficient, 0.9; $P = .0001$. (D) Correlation between IFN gamma levels and percentage of T-bet+ lamina propria cells was evaluated in 24-hour explant cultures treated with IFN alpha (500 U/mL). Spearman coefficient, 0.7; $P = .0006$. (E) Relation between IFN gamma low/high levels and T-bet+ low/high percentages was determined based on cut-off values of 30 pg/mL for IFN gamma levels and 10% for T-bet+ cells. Fisher exact test, $P = .0004$. (F) Assessment of T-bet expression by immunohistochemistry in IFN alpha-treated explants (500 U/mL, 24 hours). One representative case with high numbers of T-bet+ lamina propria lymphocytes (brown nuclei). Nuclei are counterstained in blue. AC, apoptotic crypts.

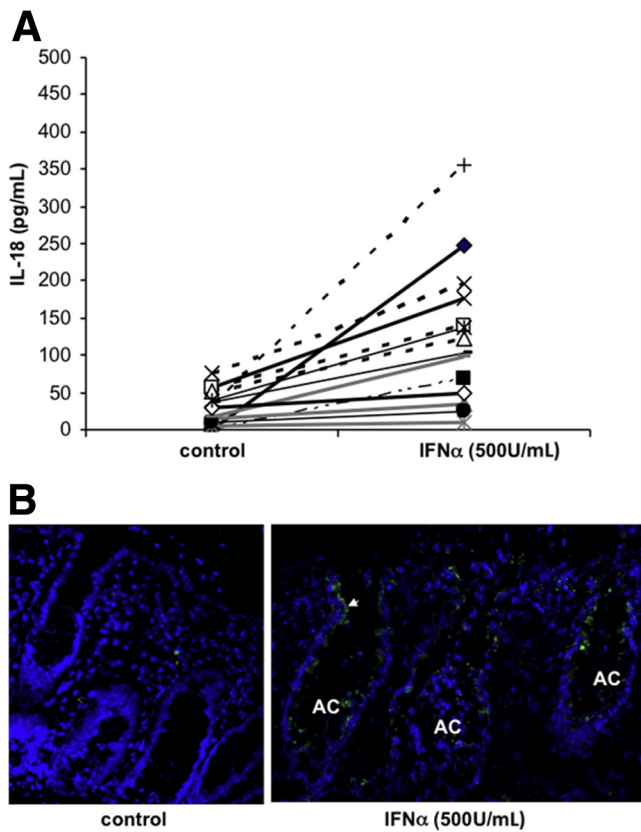


Figure 3. IFN alpha activates the caspase-1/IL18 axis in the human intestinal mucosa. (A) Measurement of mature IL18 secretion by ELISA, in the supernatants of paired control and IFN alpha-treated intestinal mucosa explant cultures from 14 individuals. Each *symbol* represents the mean value of 4 explants. The variation between the 4 explants did not exceed 20%. (B) In situ detection of active caspase-1 by the fluorescein-FLICA assay in control or IFN alpha-treated explant cultures (500 U/mL, 24 h). In control cultures, the intestinal epithelial cells were unstained; only rare lamina propria mononuclear cells were observed (green). In IFN alpha-treated explant cultures, numerous epithelial cells showed active caspase-1 (green) in apoptotic crypts (AC), present within the cytoplasm or at the apical membrane (*arrow*). Nuclei are counterstained in blue. Representative pictures of 6 different experiments.

caspase-1/IL18 axis, we conclude that IFN alpha disrupts the intestinal epithelial barrier via caspase-1 activation, followed by production of IL18 and subsequent production of IFN gamma. Two experimental controls are consistent with our interpretation of the causal role of IL18: first, IL18-BP had no deleterious effect on the intestinal crypts when used alone; second, IL18-BP had no effect on the crypt apoptosis elicited by IFN gamma (Figure 4C).

Involvement of DKK1 in the Pro-apoptotic Effect of IFN Alpha on the Intestinal Epithelial Barrier

IFN gamma has been reported to modulate intestinal epithelial homeostasis via induction of the Wnt inhibitor DKK1.²¹ We thus examined the following: first, the level of DKK1 production by ELISA, as well as the possible

correlation between DKK1 and IFN gamma levels, in IFN alpha-treated explants; and, second, the effect of neutralizing anti-DKK1 antibodies on IFN alpha-induced crypt apoptosis. As shown in Figure 5A, variable levels of DKK1 were produced by IFN alpha-treated mucosa explant cultures. There was a statistically significant correlation between the levels of DKK1 and IFN gamma produced in a given mucosa ($P = .034$; Spearman correlation coefficient, 0.67) (Figure 5A). Interestingly, the use of neutralizing anti-DKK1 antibodies in IFN alpha-treated explant cultures led to a significant decrease in the number of M30+ apoptotic crypts ($P = .01$ vs IFN alpha alone) (Figure 5B).

Discussion

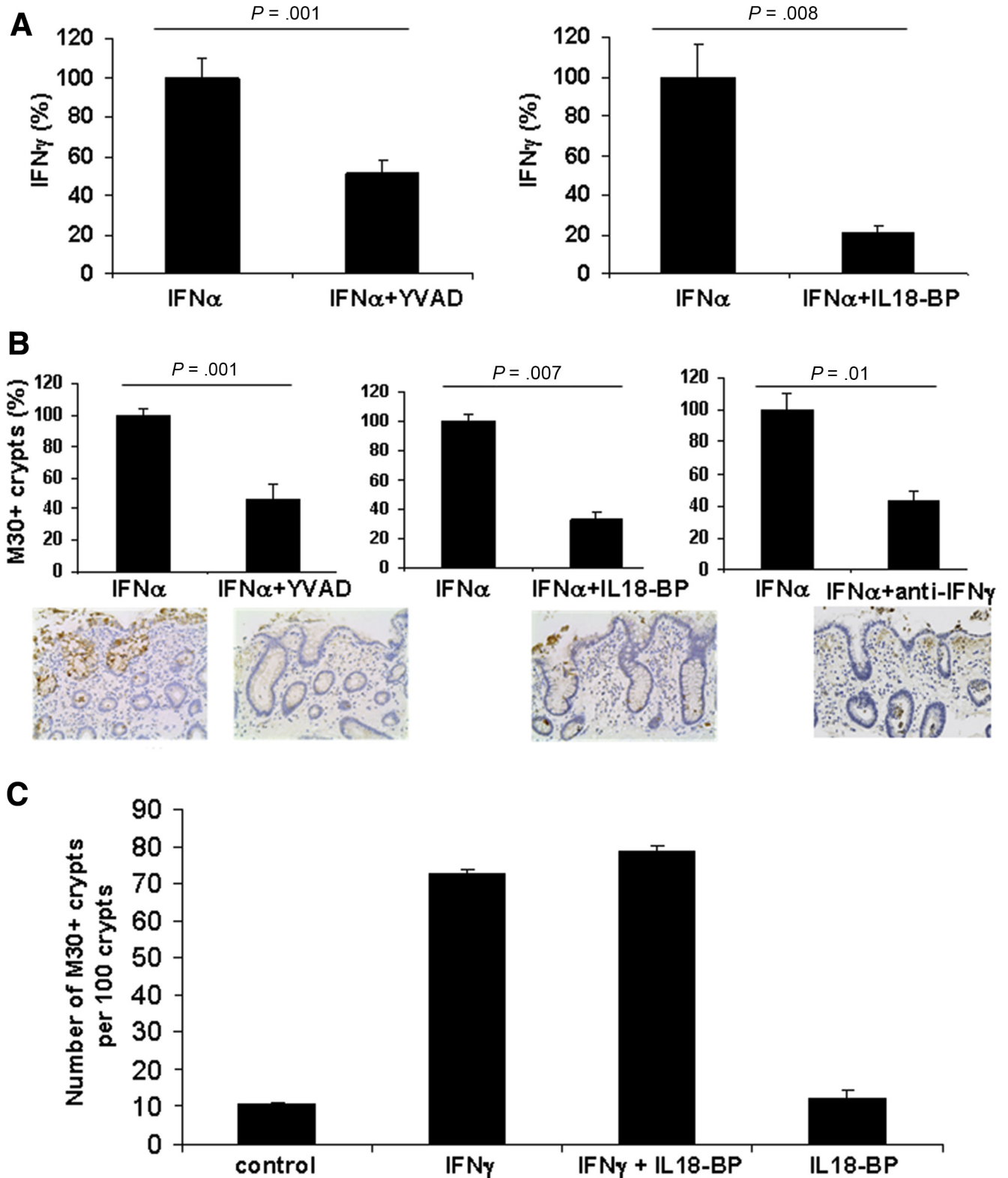
Recent reports underscore the need for deciphering the complex interactions involving mediators and specialized cell types that maintain human intestinal homeostasis.^{16,22–26} The current study aimed to decipher the mechanisms of IFN alpha action on the human adult normal mucosa homeostasis, in ex vivo explant cultures, an integrated model system maintaining the 3D architecture of the mucosa, which allows us to highlight potential cellular cross-talk between mucosal resident cells through diverse cytokines.^{15,16} We provide here several findings showing the following: (1) IFN alpha impairs human intestinal mucosa homeostasis by eliciting epithelial barrier disruption via apoptosis, (2) the IFN alpha-elicited impairment of intestinal mucosa homeostasis is heterogenous among individuals, and (3) the pro-apoptotic effect of IFN alpha on the intestinal epithelial barrier is subordinated to the caspase-1/IL18/IFN gamma axis and involves the Wnt/ β -catenin pathway.

We show that IFN alpha induces a rapid (within 24 hours) alteration of the human normal intestinal epithelial barrier with the presence of apoptotic bodies within the crypts and epithelial cells that shed at the surface and crypt base. Immunostaining with M30 antibody, which recognizes a cytokeratin-18 neo-epitope that becomes available at an early caspase cleavage event during apoptosis,^{17,18} confirmed that IFN alpha elicits epithelial apoptosis of the entire crypts (no surface to base of the crypt gradient). Noticeably, this IFN alpha-elicited apoptotic effect is variable among individuals. In fact, some intestinal mucosae can be considered as refractory to IFN alpha, and others as sensitive to IFN alpha, with a variable degree of sensitivity (15%–60% apoptotic crypts). This ex vivo finding parallels clinical observations of the interpatient variability of Roferon therapy side effects, which can be significant or negligible in patient subpopulations and often dependent on the dose, route, and schedule of administration.¹⁴ It has been reported that approximately 60% of patients with chronic hepatitis or cancer treated with Roferon have intestinal disorders, especially diarrhea. Case reports also note rare cases of pseudo-inflammatory bowel disease subsequent to IFN alpha/ribavirin treatment for chronic hepatitis.^{27,28}

Next, knowing that type I IFN often can exert their effects indirectly via the production of cytokines, we sought to determine the potential driver of the IFN alpha-induced intestinal barrier disruption. We focused on IFN gamma

because IFN alpha is known to have immunomodulatory functions and to generate a Th1 response. For example, pegylated IFN alpha 2a treatment in patients with hepatitis C virus infection induced a transient increase of serum IFN

gamma levels.²⁹ IFN alpha favors the production of IFN gamma by peripheral blood mononuclear cells or natural killer cells.^{30,31} Our intestinal mucosa explant culture model is best suited to examine the cascade of events involved in



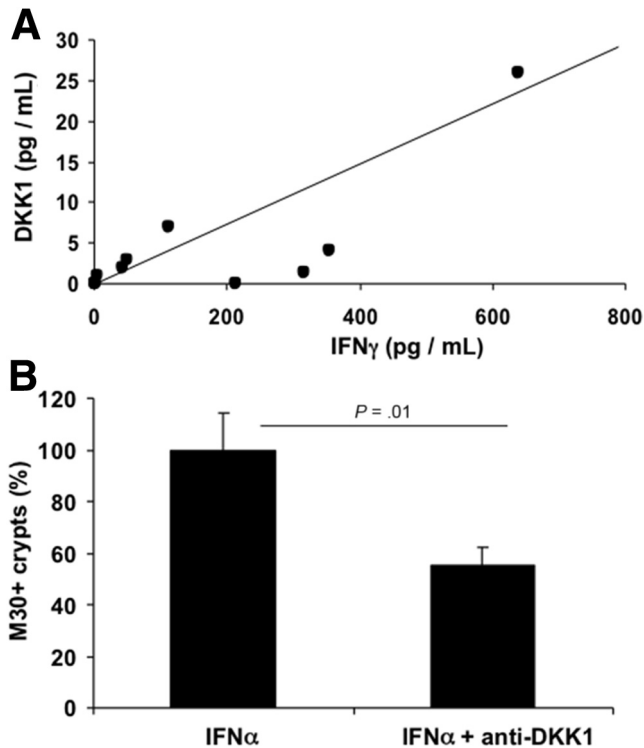


Figure 5. Involvement of the Wnt pathway in the apoptotic effect of IFN alpha on the human intestinal barrier. (A) Intestinal mucosa explant cultures were treated with 500 U/mL IFN alpha for 24 hours. Levels of the Wnt inhibitor DKK1 was assessed by ELISA in the culture supernatants of mucosae from 9 individuals and compared with the levels of IFN gamma induced by IFN alpha ($P = .034$; Spearman correlation coefficient, 0.67). Each circle represents the mean of 4 explants. The variation between the 4 explants did not exceed 20%. (B) Explant cultures were treated with IFN alpha (500 U/mL, 24 h) in the absence or presence of neutralizing anti-DKK1 antibodies (5 μ g/mL). The number of M30+ apoptotic crypts were counted and results were expressed as the percentage of apoptotic crypts with IFN alpha alone (100%). Means \pm SEM of 4 experiments.

the IFN alpha effect on the epithelial barrier because it possesses all the cell subsets and maintains the cellular interactions required for the onset of a Th1 response.^{15,16} We show here that IFN alpha led to increased numbers of T-bet+ (Th1/Tc1) lamina propria T lymphocytes in IFN alpha-sensitive mucosae, which correlated with IFN gamma

levels released in the explant cultures supernatants. Noticeably, IFN gamma levels induced by IFN alpha correlated with the percentage of M30+ apoptotic crypts in a given mucosa. Most interestingly, we show here that the IFN alpha-induced epithelial apoptosis is dependent on IFN gamma production because it is inhibited significantly by neutralizing anti-IFN gamma antibodies. Interestingly, although a direct effect of IFN alpha on epithelial cells cannot be excluded, based on reductionist approaches using porcine renal epithelial cells³² or a human intestinal colonic cancer cell line,³³ the advantage of an integrated explant culture model is to decipher the sequence of interactions between the various resident cell types of the human mucosa at the tissue level.

Furthermore, we and others have shown that IFN gamma production in the intestinal mucosa can be subordinated either to IL12 or IL18 pathways.^{16,34,35} Preliminary experiments in the explant culture model indicated that very low levels of IL12 (not shown) and much higher levels of IL18 were produced upon IFN alpha treatment, so we focused on the caspase-1/IL18 pathway, part of the inflammasome. The inflammasome is a multiprotein complex containing the central effector protein caspase-1.³⁶ Activation of caspase-1 leads to the maturation and secretion of the proinflammatory cytokine IL18, a potent stimulator of a Th1 response.³⁷ It should be noted that the human normal intestinal mucosa is a reservoir of pro-caspase-1 and pro-IL18, present in epithelial cells and in some lamina propria macrophages, which can be activated rapidly and can initiate a Th1 response.¹⁶ In addition, in recent reports, using in vitro models or mouse models, including genetically engineered mice, type I IFN has emerged as a regulator of inflammasome activation, although the exact mechanism is not understood.³⁸⁻⁴⁰

In this study, the intestinal mucosa explant culture model coupled with the use of blockers of the inflammasome pathway (caspase-1 inhibitor YVAD and IL18 antagonist IL18-BP), shows the following events: IFN alpha activates caspase-1 in epithelial cells and in some lamina propria mononuclear cells, as assessed in situ by the FLICA assay, resulting in the release of mature IL18 by these cells and in the subsequent production of IFN gamma by lamina propria T lymphocytes, which causes epithelial barrier disruption. Interestingly, these effects were found to be heterogeneous among individuals, a finding that recapitulates the interpatient variability of IFN alpha side effects in the clinic. This

Figure 4. (See previous page). The IFN alpha-induced alteration of intestinal mucosa homeostasis is subordinated to the caspase-1/IL18 axis. Intestinal mucosa explant cultures were treated with IFN alpha (500 U/mL) for 24 hours, in the absence or presence of the (A and B) caspase-1-specific inhibitor YVAD-FMK (2 μ mol/L) or IL18-BP (800 ng/mL) or (B) neutralizing anti-IFN gamma antibodies (2 μ g/mL) (right). (A) IFN gamma secretion was measured by ELISA in the culture supernatants. Results are expressed as the percentage of IFN gamma secreted by explant cultures treated with IFN alpha alone (100%). (B and C) The percentage of apoptotic crypts was evaluated after M30 immunolabeling. Results are expressed as the percentage of M30+ apoptotic crypts in IFN alpha-treated explants (100%). Means \pm SEM of 3 (anti-IFN gamma) to 4 (YVAD, anti-IL18-BP) experiments, with 4 explants per condition. (B) Pictures below histograms show representative M30 immunostainings in the various experimental conditions mentioned above the figures (apoptotic crypts in brown, nuclei counterstained in blue). (C) Control experiment to assess that IL18-BP (800 ng/mL) has no effect when used alone and no effect on crypt apoptosis (M30+ crypts) elicited by IFN gamma (50 ng/mL) in 24-hour colonic mucosa explant cultures. Means \pm SEM of 4 explants.

interindividual heterogeneity of response to IFN alpha in noninflammatory conditions could be related to interindividual differences in the genetic background. Importantly, the caspase-1-IL18-IFN gamma axis recently also was shown to be activated in Crohn's disease cases.⁴¹ In the context of the present study, one can speculate that a causal relationship exists between IFN alpha production and the activation of the inflammasome in Crohn's disease.

IFN gamma is well known to modulate intestinal epithelial barrier functions such as epithelial permeability via the disassembly and/or down-regulation of tight junction proteins, epithelial polarity, and secretory functions.⁴²⁻⁴⁵ In addition, a recent report concluded that IFN gamma is able to regulate the intestinal epithelial cell homeostatic functions (ie, cell proliferation/apoptosis through the Wnt/ β -catenin pathway). Indeed, IFN gamma up-regulates the Wnt inhibitor DKK1.²¹ We show a significant correlation between IFN gamma and DKK1 levels upon IFN alpha treatment, and of a DKK1-dependent apoptotic effect upon IFN alpha treatment, which allows to add the Wnt/ β -catenin pathway as another signaling pathway involved in the indirect effect of IFN alpha on the intestinal epithelial barrier.

Altogether, this study, using a 3D model of human normal intestinal mucosa explant culture, provides some clues on the mechanisms involved in the intestinal immunopathologic effects of IFN alpha. Indeed, this study showed that the innate cytokine IFN alpha can activate adaptive immune responses (ie, a Th1/Tc1 IFN gamma response), in normal human intestinal mucosa, which is the driver of epithelial barrier disruption, although these effects are variable among individuals and strongly suggest that the intestinal side effects of Roferon therapy are inherent to IFN gamma production, which has been shown to occur rapidly upon Roferon treatment. Interestingly, our study also showed that IFN alpha activates the inflammasome, a molecular platform present in numerous cell types and the IL18/IFN gamma axis.

References

- Trinchieri G. Type I interferon: friend or foe? *J Exp Med* 2010;207:2053-2063.
- Gonzalez-Navajas JM, Lee J, David M, et al. Immunomodulatory functions of type interferons. *Nat Rev Immunol* 2012;12:125-135.
- Hervas-Stubbs S, Perez-Gracia JL, Rouzaut A, et al. Direct effects of type I interferons on cells of the immune system. *Clin Cancer Res* 2011;17:2619-2627.
- Monteleone G, Pender SL, Wathen NC, et al. Interferon-alpha drives T cell-mediated immunopathology in the intestine. *Eur J Immunol* 2001;31:2247-2255.
- Di Sabatino A, Pickard KM, Gordon JN, et al. Evidence for the role of interferon-alfa production by dendritic cells in the Th1 response in celiac disease. *Gastroenterology* 2007;133:1175-1187.
- Giles EM, Sanders TJ, McCarthy NE, et al. Regulation of human intestinal T-cell responses by type 1 interferon-STAT1 signaling is disrupted in inflammatory bowel disease. *Mucosal Immunol* 2016, Epub ahead of print.
- Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci U S A* 1994; 91:1198-1205.
- Brassard DL, Grace MJ, Bordens RW. Interferon-alpha as an immunotherapeutic protein. *J Leukoc Biol* 2002; 71:565-581.
- Gresser I, Kaido T, Maury C, et al. Interaction of IFN alpha/beta with host cells essential to the early inhibition of Friend erythroleukemia visceral metastases in mice. *Int J Cancer* 1994;57:604-611.
- Kaido T, Maury C, Schirmmacher V, et al. Successful immunotherapy of the highly metastatic murine ESb lymphoma with sensitized CD8+ T cells and IFN-alpha/beta. *Int J Cancer* 1994;57:538-543.
- Kaido TJ, Maury C, Gresser I. Host CD4+ T lymphocytes are required for the synergistic action of interferon-alpha/beta and adoptively transferred immune cells in the inhibition of visceral ESb metastases. *Cancer Res* 1995; 55:6133-6139.
- Madera S, Rapp M, Firth MA, et al. Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. *J Exp Med* 2016; 213:225-233.
- Cangemi G, Millo E, Damonte G, et al. alpha-Interferon treatment induces quantitative modifications of HLA class I-associated peptides eluted from cultured cancer cell lines. *Tissue Antigens* 2000;55:212-218.
- Sleijfer S, Bannink M, Van Gool AR, et al. Side effects of interferon-alpha therapy. *Pharm World Sci* 2005; 27:423-431.
- 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, Sarrabayrouse 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; 141:1887-1896, e1-2.
- Caulin C, Salvesen GS, 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, Kolgen W, Bjorklund 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.
- Sullivan BM, Juedes A, Szabo SJ, et al. Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc Natl Acad Sci U S A* 2003;100:15818-15823.
- Nava P, Koch S, Laukoetter MG, et al. Interferon-gamma regulates intestinal epithelial homeostasis through converging beta-catenin signaling pathways. *Immunity* 2010;32:392-402.
- Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* 2014;14:141-153.

23. Fang K, Sideri A, Law IK, et al. Identification of a novel substance P (SP)-neurokinin-1 receptor (NK-1R)micro-RNA-221-5p inflammatory network in human colonic epithelial cells. *Cell Mol Gastroenterol Hepatol* 2015; 1:503–515.
24. Mishima Y, Liu B, Hansen JJ, et al. Resident bacteria-stimulated IL-10-secreting B cells ameliorate T cell-mediated colitis by inducing Tr-1 cells that require IL-27-signaling. *Cell Mol Gastroenterol Hepatol* 2015; 1:295–310.
25. Weinhage T, Däbritz J, Brockhausen A, et al. Granulocyte macrophage colony-stimulating factor-activated CD39+/CD73+ murine monocytes modulate intestinal inflammation via induction of regulatory T cells. *Cell Mol Gastroenterol Hepatol* 2015;1:433–449.
26. Singh V, Yeoh BS, Chassaing B, et al. Microbiota-inducible innate immune, siderophore binding protein lipocalin 2 is critical for intestinal homeostasis. *Cell Mol Gastroenterol Hepatol* 2016;2:482–498.
27. Tada H, Saitoh S, Nakagawa Y, et al. Ischemic colitis during interferon-alpha treatment for chronic active hepatitis C. *J Gastroenterol* 1996;31:582–584.
28. Sprenger R, Sagmeister M, Offner F. Acute ulcerative colitis during successful interferon/ribavirin treatment for chronic hepatitis. *Gut* 2005;54:438–439.
29. Freeman J, Baglino S, Friborg J, et al. Pegylated interferons lambda-1a and alfa-2a display different gene induction and cytokine and chemokine release profiles in whole blood, human hepatocytes and peripheral blood mononuclear cells. *J Viral Hepat* 2014;21:e1–e9.
30. Brinkmann V, Geiger T, Alkan S, et al. Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. *J Exp Med* 1993;178:1655–1663.
31. Marshall DJ, San Mateo LR, Rudnick KA, et al. Induction of Th1-type immunity and tumor protection with a prostate-specific antigen DNA vaccine. *Cancer Immunol Immunother* 2005;54:1082–1094.
32. Lechner J, Malloth N, Seppi T, et al. IFN-alpha induces barrier destabilization and apoptosis in renal proximal tubular epithelium. *Am J Physiol Cell Physiol* 2008; 294:C153–C160.
33. Frias AH, Jones RM, Fifadara NH, et al. Rotavirus-induced FN- β promotes anti-viral signaling and apoptosis that modulate viral replication in intestinal epithelial cells. *Innate Immun* 2012;18:294–306.
34. Siegmund B, Fantuzzi G, Rieder F, et al. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am J Physiol Regul Integr Comp Physiol* 2011;281:R1264–R1273.
35. Strober W, Zhang F, Kitani A, et al. Proinflammatory cytokines underlying the inflammation of Crohn's disease. *Curr Opin Gastroenterol* 2010;26:310–317.
36. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol* 2009; 27:229–265.
37. Dinarello CA. Interleukin-18. *Methods* 1999;19:121–132.
38. Freudenberg MA, Merlin T, Kalis C, et al. Cutting edge: a murine, IL-12-independent pathway of IFN-gamma induction by gram-negative bacteria based on STAT4 activation by type I IFN and IL-18 signaling. *J Immunol* 2002;169:1665–1668.
39. Pothlichet J, Meunier I, Davis BK, et al. Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in influenza A virus infected cells. *PLoS Pathog* 2013;9:e1003256.
40. Yang Q, Stevenson HL, Scott MJ, et al. Type I interferon contributes to noncanonical inflammasome activation, mediates immunopathology, and impairs protective immunity during fatal infection with lipopolysaccharide-negative ehrlichiae. *Am J Pathol* 2015;185:446–461.
41. Jarry A, Bossard C, Droy-Dupré L, et al. Heterogeneity of subordination of the IL-18/IFN- γ axis to caspase-1 among patients with Crohn's disease. *Lab Invest* 2015; 95:1207–1217.
42. Madara JL, Stafford J. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. *J Clin Invest* 1989;83:724–727.
43. Bruewer M, Luegering A, Kucharzik T, et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *J Immunol* 2003;171:6164–6172.
44. Wang F, Graham WV, Wang Y, et al. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. *Am J Pathol* 2005;166:409–419.
45. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009;9:799–809.

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Conflicts of interest

The authors disclose no conflicts.

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