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Research Article

Gastroenterology

IL-10 is an immunomodulatory cytokine that plays an obligate role in preventing spontaneous enterocolitis in mice. However, little is known about IL-10 function in the human intestinal mucosa. We showed here that IL-10 was constitutively expressed and secreted by the human normal colonic mucosa, including epithelial cells. Depletion of IL-10 in mucosal explants induced both downregulation of the IL-10-inducible, immunosuppressive gene *BCL3* and upregulation of IFN- $\gamma$ , TNF- $\alpha$ , and IL-17. Interestingly, TGF- $\beta$  blockade also strongly induced IFN- $\gamma$  production. In addition, the high levels of IFN- $\gamma$  produced upon IL-10 depletion were responsible for surface epithelium damage and crypt loss, mainly by apoptosis. Polymyxin B, used as a scavenger of endogenous LPS, abolished both IFN- $\gamma$  production and epithelial barrier disruption. Finally, adding a commensal bacteria strain to mucosa explant cultures depleted of both IL-10 and LPS reproduced the ability of endogenous LPS to induce IFN- $\gamma$  secretion. These findings demonstrate that IL-10 ablation leads to an endogenous IFN- $\gamma$ -mediated inflammatory response via LPS from commensal bacteria in the human colonic mucosa. We also found that both IL-10 and TGF- $\beta$  play crucial roles in maintaining human colonic mucosa homeostasis.

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# Mucosal IL-10 and TGF- $\beta$ play crucial roles in preventing LPS-driven, IFN- $\gamma$ -mediated epithelial damage in human colon explants

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**IL-10 is an immunomodulatory cytokine that plays an obligate role in preventing spontaneous enterocolitis in mice. However, little is known about IL-10 function in the human intestinal mucosa. We showed here that IL-10 was constitutively expressed and secreted by the human normal colonic mucosa, including epithelial cells. Depletion of IL-10 in mucosal explants induced both downregulation of the IL-10-inducible, immunosuppressive gene *BCL3* and upregulation of IFN- $\gamma$ , TNF- $\alpha$ , and IL-17. Interestingly, TGF- $\beta$  blockade also strongly induced IFN- $\gamma$  production. In addition, the high levels of IFN- $\gamma$  produced upon IL-10 depletion were responsible for surface epithelium damage and crypt loss, mainly by apoptosis. Polymyxin B, used as a scavenger of endogenous LPS, abolished both IFN- $\gamma$  production and epithelial barrier disruption. Finally, adding a commensal bacteria strain to mucosa explant cultures depleted of both IL-10 and LPS reproduced the ability of endogenous LPS to induce IFN- $\gamma$  secretion. These findings demonstrate that IL-10 ablation leads to an endogenous IFN- $\gamma$ -mediated inflammatory response via LPS from commensal bacteria in the human colonic mucosa. We also found that both IL-10 and TGF- $\beta$  play crucial roles in maintaining human colonic mucosa homeostasis.**

## Introduction

It is largely unknown how the colonic mucosa can avoid overt inflammation, given that a single layer of polarized epithelial cells separates it from the luminal microflora, which possesses molecules in common with various pathogens. It was only when spontaneous enterocolitis was observed in genetically modified mouse strains that the importance of a finely tuned balanced immune response for maintaining intestinal tissue homeostasis was fully appreciated (1).

The finding that IL-10-deficient mice develop an inflammation restricted to the intestine points to the important immunoregulatory role of IL-10 at this site (2–5). In addition, studies using T cell-restored SCID mice that develop a severe inflammatory response in the colon have provided evidence that IL-10 plays an obligate role in the function of regulatory T cells that inhibit inflammatory responses in the intestine (6). Interestingly, colitis, both in IL-10-deficient mice and in the SCID model, involves the development of Th1 cells responding primarily to intestinal flora (7). Together these studies support the concept that in immunocompetent hosts, enteric antigens, probably of bacterial origin, induce IL-10-secreting T cells that are immune suppressive and prevent colitis.

Whether this concept can also account for the colonic mucosal homeostasis in humans remains unknown. Because of the lack of suitable model systems, the immunosuppressive role of IL-10 in the human intestine is largely conjectural. In fact, clinical studies have shown that systemically administered IL-10 to patients with inflammatory bowel disease has a tendency toward clinical

improvement, but not remission, and can even trigger and amplify a Th1 inflammatory response (8–11). These studies raise the important issue of whether systemic IL-10 has different effects from that produced locally.

In this study, we addressed the issue of the main sources of IL-10 in the human colon and of its endogenous function in the intestinal mucosa. We identified the epithelial lining of the human colon, strategically located at the interface between the luminal content and the mucosa, as an important source of mucosal IL-10. We analyzed the role of IL-10 in maintaining mucosal homeostasis using an approach based on mucosal IL-10 depletion in explant cultures of colonic mucosa, using neutralizing anti-IL-10 antibodies. In addition, we examined the effects of neutralizing another important immunoregulatory cytokine, TGF- $\beta$ , in explant cultures. We present evidence that both mucosal IL-10 and TGF- $\beta$  are critical for maintaining human colonic mucosa integrity.

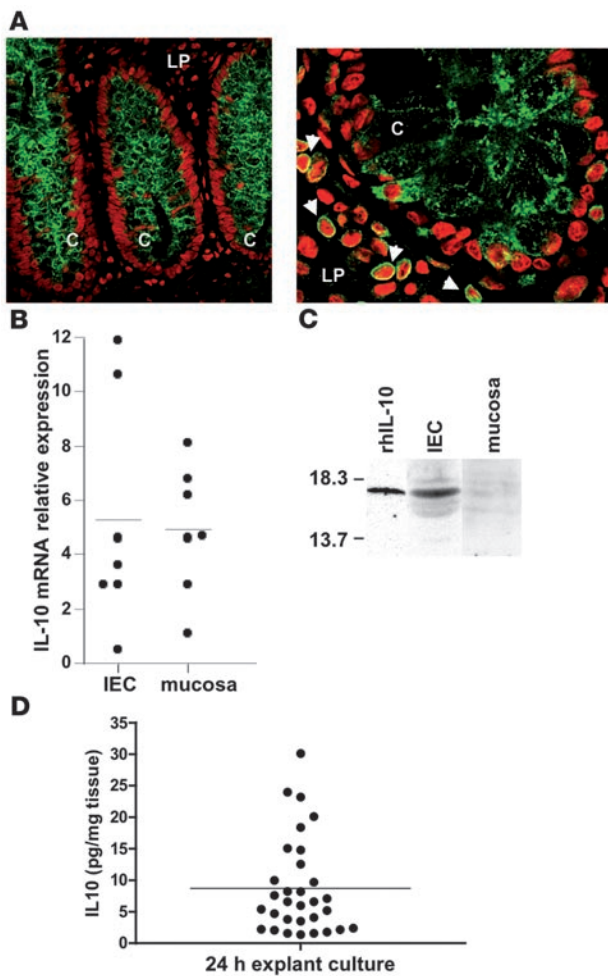
## Results

**IL-10 production by the human colonic mucosa.** Immunofluorescence followed by confocal microscopy of normal human colonic mucosa showed IL-10 expression by epithelial cells, both in the surface epithelium and at the base of the crypts, and by a few lamina propria mononuclear cells (Figure 1A). IL-10 production by epithelial cells was then confirmed at the mRNA level using real-time PCR on preparations of epithelial cells (Figure 1B). Immunoblot analysis using the anti-IL-10 antibody showed a strong band of the same molecular weight as that of recombinant human IL-10 (rhIL-10) in the lysates from isolated epithelial cells, confirming the specificity of the anti-IL-10 antibody (Figure 1C). A faint band was observed in the lysates from whole mucosa, in line with our immunofluorescence studies, which suggests that epithelial cells are an important source of IL-10

**Nonstandard abbreviations used:** rh-, recombinant human; TGF- $\beta$ RII, TGF- $\beta$  type II receptor.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

Expression and secretion of IL-10 by the human colonic mucosa. **(A)** In situ expression of IL-10 in the human colonic mucosa, visualized by immunofluorescence staining with anti-IL-10 monoclonal antibodies and confocal microscopy (see Methods). IL-10 (green) is mainly expressed by the epithelial cells of the crypts (C) and by a few mononuclear cells (arrows) in the lamina propria (LP). Nuclei appear in red. Original magnification,  $\times 630$  (left);  $\times 1,000$  (right). **(B)** Real-time PCR analysis of IL-10 mRNA levels, expressed relative to  $\beta$ -actin levels (see Methods), in preparations of isolated human intestinal epithelial cells (IEC) or of matching whole mucosa microdissected from normal colon. Horizontal lines represent mean value of 7 determinations from 4 patients. **(C)** Immunoblot analysis of IL-10 was performed as described in Methods from lysates of isolated human intestinal epithelial cells or of whole mucosa microdissected from normal colon. Shown is 1 representative experiment of 3. Numbers at left represent molecular size of standards in kDa. **(D)** IL-10 secretion, measured by ELISA, in 24-h explant cultures of human normal colonic mucosa (see Methods). Data are expressed as pg/mg wet tissue. Each point indicates individual explant culture; horizontal line represents mean value of 31 determinations from 8 independent experiments.

In line with the results obtained at the mRNA level, the amounts of IFN- $\gamma$  and TNF- $\alpha$  protein levels, as measured by ELISA in the supernatants, were very low in control cultures (Figure 3B). At 48 h, in IL-10-depleted cultures an 11-fold increase in IFN- $\gamma$  and a 5-fold increase in TNF- $\alpha$  protein levels were observed compared with control cultures (Figure 3B).

The level of IL-17, recently described to play a role in several models of experimental colitis (see Discussion), was determined by ELISA in the supernatants of 48-h cultures and was found to be 2-fold higher in IL-10-depleted mucosa explant cultures compared with control cultures (Figure 4A). In these experiments, IFN- $\gamma$  levels were 10-fold higher in IL-10-depleted mucosa explant cultures than in control cultures (Figure 4B).

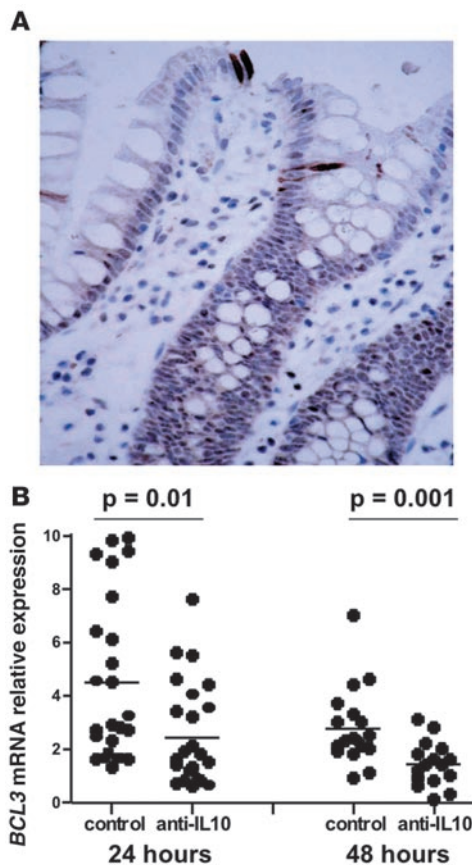
*TGF- $\beta$  blockade also elicited an IFN- $\gamma$  response.* We next addressed the issue of whether TGF- $\beta$ , another important antiinflammatory cytokine, has homeostatic properties in this explant culture model. Our approach was to treat mucosa explant cultures for 48 h with neutralizing anti-TGF- $\beta$  type II receptor (anti-TGF- $\beta$ RII) antibodies, known to inhibit the effects of TGF- $\beta$ s (13). As shown in Figure 5, A and B, TGF- $\beta$  blockade induced a strong IFN- $\gamma$  response compared with control cultures, both at the mRNA and at the protein level. In these experiments, IL-10 mRNA levels remained unchanged (Figure 5C). Adding rhTGF- $\beta$ 1 (10 ng/ml) to 48-h IL-10-depleted mucosa explant cultures did not significantly modify IFN- $\gamma$  levels (Figure 5D). Finally, TGF- $\beta$  mRNA levels were not modified upon IL-10 depletion (Figure 5E).

*Epithelial barrier disruption in IL-10-depleted mucosa.* At 24 h of control culture, mucosal histology was well preserved and similar to that observed before culture (Figure 6A). In IL-10-depleted mucosa at 24 h, the epithelial cells of both surface epithelium and at the base of the crypts were well polarized and differentiated; irregular branching of some crypts was sometimes observed (Figure 6A), but the number of crypts counted on paraffin sections was similar in IL-10-depleted and control mucosa (data not shown). At 48 h of control culture we observed patchy areas of surface epithelial irregularity as well as crypt distortion and variability in crypt diameter; in IL-10-depleted mucosa at 48 h, the mucosa was thinner than in controls, and the surface epithelium as well as the base of the crypts were highly damaged, with a loss

in the human colonic mucosa. Finally, as measured by ELISA, IL-10 was secreted in the supernatant of 24-h cultures of human colonic mucosa, although at variable levels among the samples studied (Figure 1D).

*Validation of an experimental model of IL-10 depletion in the human colonic mucosa using neutralizing anti-IL-10 antibodies.* To verify that IL-10 depletion was achieved with neutralizing anti-IL-10 antibodies, we examined the expression of *BCL3*, an IL-10-inducible, immunosuppressive gene (12). In the human colonic mucosa, *BCL3* was highly expressed by epithelial cells and a few lamina propria mononuclear cells (nuclear expression) (Figure 2A). IL-10 depletion in 24- and 48-h mucosa explant cultures led to significant downregulation of *BCL3* mRNA levels compared with isotype IgG1 controls, as assessed by real-time PCR (Figure 2B). This result validated the use of neutralizing anti-IL-10 antibodies as an effective means of depleting intramucosal IL-10.

*Intramucosal IL-10 depletion in the human colonic mucosa led to an induction of IFN- $\gamma$  production.* We next examined the effect of intramucosal IL-10 depletion on the expression of 2 proinflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , in mucosa explant cultures at 24 and 48 h, both at the mRNA and protein levels. In control cultures, IFN- $\gamma$  and TNF- $\alpha$  mRNA levels remained very low at both 24 and 48 h (Figure 3A). Interestingly, at 48 h, a 13-fold increase in IFN- $\gamma$  and a 2-fold increase in TNF- $\alpha$  mRNA levels were observed in IL-10-depleted cultures compared with control cultures (Figure 3A).



**Figure 2**

Validation of IL-10 depletion in colonic mucosa explants by neutralizing antibodies: decrease in *BCL3*, an IL-10-inducible gene. **(A)** *BCL3* immunohistochemistry on paraffin sections of human normal colonic mucosa. *BCL3* was present in the nuclei of the majority of epithelial cells (brown) and in a few lamina propria mononuclear cells. Hematoxylin counterstaining. Original magnification,  $\times 400$ . **(B)** Relative expression of *BCL3* mRNA levels in 24- and 48-h mucosa explant cultures, incubated with anti-IL-10 neutralizing monoclonal antibodies (1  $\mu\text{g/ml}$ ) or with isotype mouse IgG1 (control). Total RNA was isolated from each sample and reverse transcribed. *BCL3* and  $\beta$ -actin mRNAs were quantified by real-time PCR (see Methods), and the *BCL3*/ $\beta$ -actin ratio was calculated. Each point represents the mean of duplicate measurements for 1 sample. Horizontal lines represent mean values of 6 different experiments at 24 h and 5 experiments at 48 h.

of crypts (Figure 6B). The number of remaining crypts counted on paraffin sections in IL-10-depleted mucosa was significantly decreased compared with that of control cultures (Figure 6C). In IL-10-depleted mucosa at 48 h, numerous exfoliated epithelial cells scored positive with the M30 antibody, with the TUNEL assay, and with an antibody against activated caspase-3 (Figure 7), showing that epithelial cell death was apoptotic.

*IFN- $\gamma$  was involved in the epithelial barrier disruption in IL-10-depleted mucosa.* Our preliminary observations that the extent of the damage to IL-10-depleted mucosa paralleled the amount of IFN- $\gamma$  secreted into the culture medium prompted us to examine whether IFN- $\gamma$  is involved in epithelial disruption. As shown in Figure 6, B and C, the addition of anti-IFN- $\gamma$  neutralizing antibodies (1  $\mu\text{g/ml}$  for 48 h) to the IL-10-depleted mucosa led to a partial protection of epithelial morphology compared with IL-10-depleted mucosa alone: the thickness of the mucosa was identical to that of control cultures; the number of crypts was 2-fold higher than in IL-10-depleted mucosa, with crypt height between that of control cultures and the IL-10-depleted mucosa; and the surface epithelium was polarized and differentiated, with patchy areas of regeneration.

*IFN- $\gamma$  production and epithelial barrier disruption depend on mucosal LPS in IL-10-depleted mucosa explant cultures.* Despite the numerous washings of the mucosa before culture, it is impossible to get rid of LPS from commensal bacteria. Indeed, LPS measurements using the Limulus assay in the spent media of 24-h cultures showed variable levels of LPS (mean,  $55 \pm 35$  U/ml; range, 0.25–296 U/ml;  $n = 11$ ). In in vivo conditions, luminal LPS is restricted to the luminal surface of intestinal epithelial cells and

has no access to the mucosa. In our ex vivo explant cultures, commensal LPS had access to the whole mucosa. We hypothesized that LPS translocation to the mucosa plays a role in IFN- $\gamma$  production. To test this hypothesis, the LPS scavenger polymyxin B (10  $\mu\text{g/ml}$ ) was added to the cultures, resulting in undetectable levels of LPS in the culture media. Polymyxin B prevented the IFN- $\gamma$  increase both at the mRNA and at the protein levels (the latter as measured by ELISA), in IL-10-depleted mucosa explant cultures at 48 h (Figure 8A). Accordingly, this inhibitory effect of polymyxin B on IFN- $\gamma$  production in IL-10-depleted mucosa was associated with the prevention of epithelial barrier disruption and crypt loss over a 48-h time period (Figure 8, B and C).

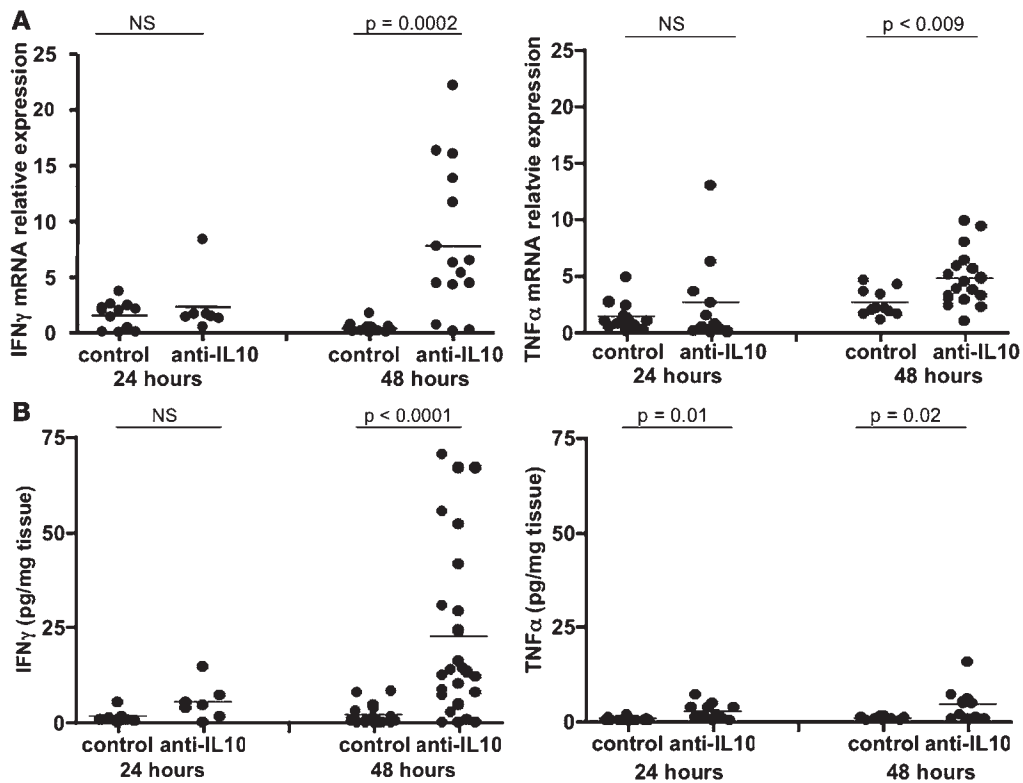
To determine whether whole commensal bacteria trigger an IFN- $\gamma$ -driven inflammatory reaction upon IL-10 depletion, mucosal explants were washed with polymyxin B to get rid of commensal bacterial LPS present in surgical resections and then exposed for 24 h to either heat-inactivated or live *E. coli* (commensal strain *E. coli* K12 MG1655) in the presence of anti-IL-10 antibodies. As shown in Figure 9, both heat-inactivated and live *E. coli* caused significant increases in IFN- $\gamma$  secretion in 24-h IL-10-depleted mucosa explant cultures compared with cultures without *E. coli* (4.5- and 6-fold increases, respectively).

## Discussion

Most of our understanding of the effects of IL-10 in humans comes from the systemic administration of IL-10 to patients with Crohn disease (8–11) or to healthy volunteers subjected to experimental endotoxemia (14). In these conditions, IL-10 can induce a proinflammatory effect with enhanced Th1 immune response (10, 11, 14). In fact, based on experimental data showing that IL-10 can be either immunosuppressive or proinflammatory, IL-10 should be considered an immunomodulatory cytokine. It is inappropriate to infer the mucosal effect of IL-10 produced in situ from experiments based on systemic administration. This issue has already been raised by Ina et al. (15), who showed, in a coculture model system, the importance of studying organ-specific cells to define the mechanisms of intestinal mucosa homeostasis.

In this context, it was important to design an experimental system allowing us to: (a) characterize the main resident cells producing IL-10 in the human intestinal mucosa; (b) delineate the crosstalk of these IL-10-producing cells with other mucosal resident cells; and (c) examine the function of the locally produced IL-10 on intestinal homeostasis. In the present study, based on microdissected human colonic mucosa that can be maintained as explant cultures, we provide several findings that



**Figure 3**

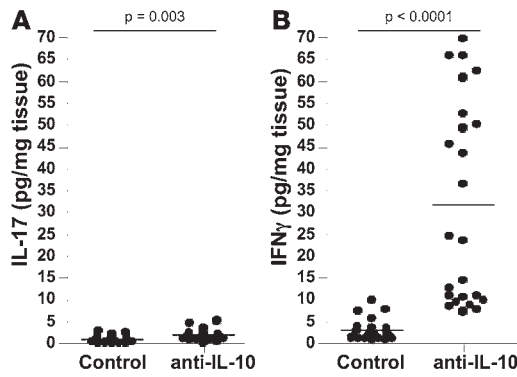
Time course of IFN- $\gamma$  and TNF- $\alpha$  production in IL-10-depleted mucosa explant cultures. Mucosa explant cultures were incubated with neutralizing anti-IL-10 antibodies or with the isotype mouse IgG1 (control) for 24 or 48 h. (A) RNA was extracted, and real-time PCR was performed. IFN- $\gamma$  (left) and TNF- $\alpha$  (right) mRNAs were quantified as described in Methods, and IFN- $\gamma$ / $\beta$ -actin and TNF- $\alpha$ / $\beta$ -actin ratios were calculated. (B) Secretion of IFN- $\gamma$  (left) and TNF- $\alpha$  (right) was measured by ELISA in the supernatant of 24- or 48-h explant cultures treated with anti-IL-10 antibodies or with the isotype mouse IgG1. Horizontal lines represent mean values of 3 different experiments at 24 h and 5 experiments at 48 h, performed in triplicate or quadruplicate.

we believe to be novel. We identified the mucosal epithelial lining as an important source of IL-10. We also showed that mucosal IL-10 depletion by neutralizing anti-IL-10 antibodies induced a strong IFN- $\gamma$  response that was responsible for the intestinal barrier disruption. Finally, we identified LPS as the main bacterial product able to trigger this inflammatory response from endogenous colitogenic cells.

First, we identified the intestinal epithelial barrier as an important source of intramucosal IL-10 by using in situ analysis of normal human colonic mucosa, real-time PCR, and immunoblot analysis of isolated epithelial cells. This finding fits well with the concept of an immunoregulatory role of the intestinal epithelium via the production of a variety of cytokines or chemokines (e.g., IL-1, IL-6, IL-18, IL-8, and TGF- $\beta$ ; refs. 16–19). Our findings are in line with those of Autschbach et al. (20) showing strong expression of IL-10 mRNA and protein by the human intestinal mucosa and mainly by the epithelial barrier, as determined using immunohistochemistry and in situ hybridization. In addition, we demonstrate in this study that IL-10 was not only produced, but also secreted, in the culture medium of mucosa explant cultures. The current prevailing concept in the literature is that IL-10 is synthesized in the human intestinal mucosa mainly by immune cells – including regulatory T cells, B cells, macrophages, and dendritic cells – and also by fibroblasts and epithelial cells (15, 20–22), a distribution analog to that previously reported in the mouse intes-

tinal mucosa (23–29). Here we show that IL-10 was constitutively synthesized and secreted in the human colonic mucosa by resident cells, including epithelial cells. It is difficult, however, to assess the relative participation of epithelial and resident immune cells in IL-10 production, because the turnover of IL-10 protein may be different in these 2 compartments. In addition, whether regulatory mechanisms of IL-10 production can occur in the epithelial compartment remains unknown, although this has been suggested by a recent report on human colonic cell lines (30).

To assess the role of IL-10 produced at the strategic interface between the intestinal lumen and the internal milieu, an in vivo approach being impossible, we developed and validated an ex vivo model system of colonic mucosa explants depleted of IL-10 using neutralizing anti-IL-10 antibodies. To validate our model, we chose BCL3, a downstream target of IL-10 (12), which is part of an immunosuppressive signaling system, as shown by BCL3 inhibition through its association with NF $\kappa$ B p50 homodimers (12, 31). Here we show, for the first time to our knowledge, that BCL3 is present within the human colonic mucosa, mainly in the nuclei of epithelial cells. IL-10 depletion in the human colonic mucosa explant cultures elicited a significant downregulation of BCL3 mRNA levels, validating the depletion of IL-10 by neutralizing anti-IL-10 antibodies. In addition, these findings suggest that BCL3 is part of the immunosuppressive machinery elicited by IL-10 in the human intestinal mucosa.

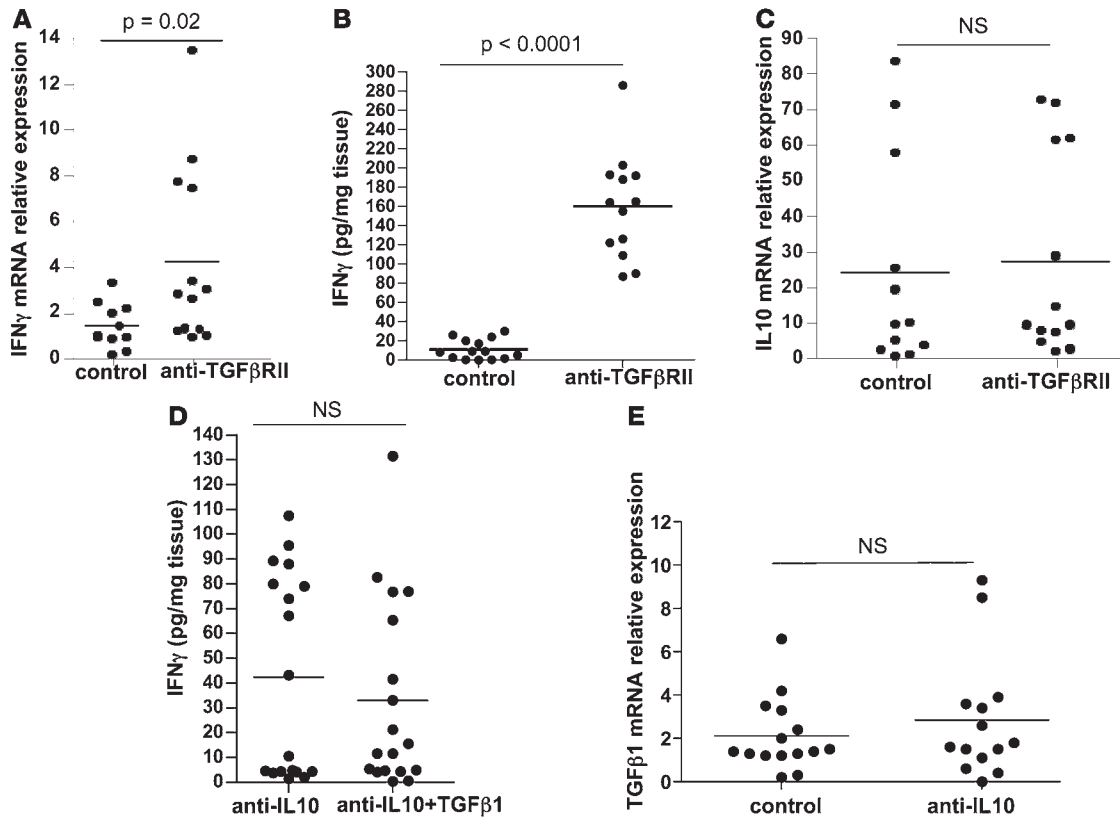


**Figure 4**

IL-17 production in IL-10–depleted mucosa explant cultures. Secretion of IL-17 (A) and IFN- $\gamma$  (B) was measured by ELISA. Horizontal lines represent mean values of 6 different experiments, in which both IL-17 and IFN- $\gamma$  were assayed in the same supernatant aliquots.

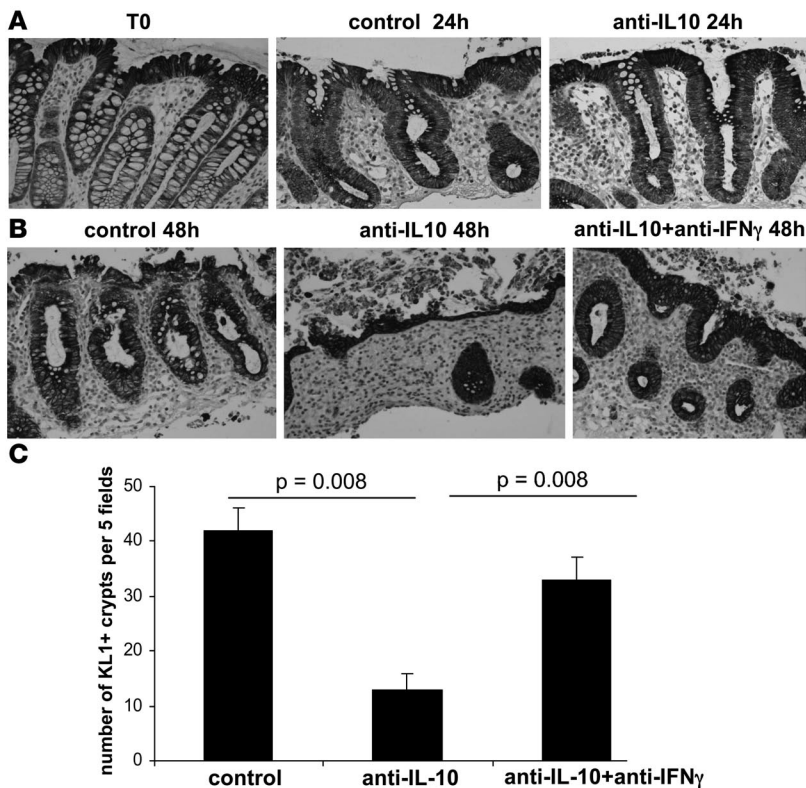
We then showed that IL-10 depletion led to an inflammatory response, monitored by the expression of TNF- $\alpha$  and IFN- $\gamma$ . We chose these Th1-type cytokines because (a) both are part of the mucosal immune response in the spontaneous colitis in IL-10<sup>-/-</sup> mice (5); (b) TNF- $\alpha$  is a major cytokine involved in the perpetuation of inflammation during inflammatory bowel diseases (32–34); and (c) both are known to induce epithelial barrier

disruption either independently or in combination (30, 35–41). We found a modest increase in TNF- $\alpha$  mRNA and protein levels in IL-10–depleted mucosa explant cultures at 48 h, in line with the partial downregulation of *BCL3* levels. This result also suggests that the very high levels of TNF- $\alpha$  reported in inflammatory bowel diseases are produced by recruited immune cells rather than by resident colitogenic cells. In contrast, strong



**Figure 5**

IFN- $\gamma$  production upon TGF- $\beta$  or IL-10 depletion. Effect of rhTGF- $\beta$  upon IL-10 depletion. (A–C) Mucosa explant cultures were incubated with neutralizing anti-TGF- $\beta$ RII antibodies (5  $\mu$ g/ml) or with control goat Ig for 48 h. IFN- $\gamma$  was examined at the mRNA (real-time PCR; A) and protein (ELISA; B) levels. IL-10 mRNA levels were also determined by real-time PCR (C). Horizontal lines represent mean values of 3 different experiments. (D and E) Mucosa explant cultures were incubated with neutralizing anti-IL-10 antibodies or with the isotype mouse IgG1 (control) for 48 h. The effect on IFN- $\gamma$  release of incubating IL-10–depleted mucosa explant cultures with rhTGF- $\beta$ 1 (10 ng/ml) was determined into the supernatants, measured by ELISA (D). TGF- $\beta$ 1 mRNA levels were quantified by real-time PCR in control and IL-10–depleted mucosa explant cultures (E). Horizontal lines represent mean values of 4 different experiments. “NS” indicates differences were not significant for the pooled experiments as well as within each experiment.

**Figure 6**

Epithelial barrier disruption in IL-10-depleted mucosa explant cultures, elicited by IFN- $\gamma$ . (A and B) Morphology of the mucosa explants before culture (T0) or after 24- or 48-h culture with or without anti-IL-10 antibodies. Immunoperoxidase staining of colonic epithelial cells with the anti-cytokeratin antibody KL-1 on paraffin sections; hematoxylin counterstaining. (A) At 24 h, the epithelial barrier was polarized and differentiated, either in control cultures or upon IL-10 depletion. (B) At 48 h, IL-10 depletion was associated with a massive epithelial barrier disruption compared with control cultures. Addition of neutralizing anti-IFN- $\gamma$  antibodies to IL-10-depleted explant cultures led to a protection of epithelial morphology. Original magnification,  $\times 200$ . (C) Counts of KL-1-positive intact crypts in 48-h mucosa explant cultures treated or not with anti-IL-10 antibodies or with both anti-IL-10 and anti-IFN- $\gamma$  antibodies (1  $\mu$ g/ml each). Values are mean  $\pm$  SEM of 4 different experiments.

IFN- $\gamma$  production was noted in IL-10-depleted mucosa explant cultures compared with control cultures at 48 h. These findings imply that resident colitogenic cells present within the lamina propria are able to secrete high amounts of IFN- $\gamma$  when intramucosal IL-10 is blocked and no longer exerts its immunosuppressive effect. In the lamina propria, it is well known that the predominant CD4 $^{+}$  mononuclear cells, and to a lesser extent CD8 $^{+}$  mononuclear cells, are IFN- $\gamma$  producers, and these cells were also present in our mucosa explant cultures (A, Jarry and C. Bossard, unpublished observation). This cytokine profile in IL-10-depleted mucosa explant cultures is reminiscent of the Th1 immune response observed in IL-10 $^{-/-}$  mice (5).

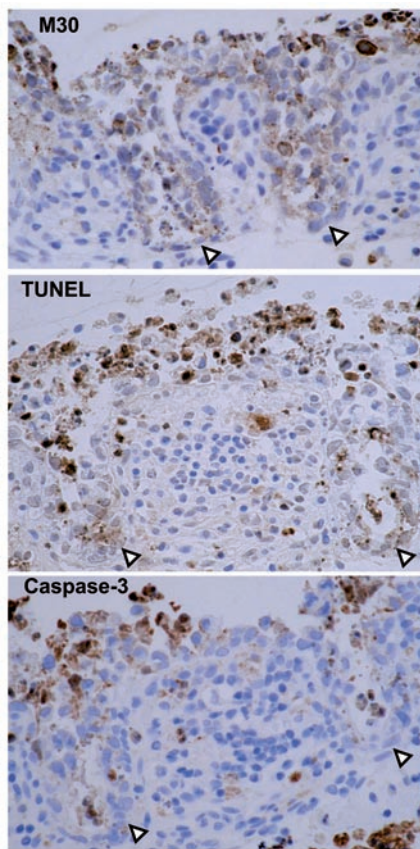
We then examined whether IFN- $\gamma$  elicits deleterious effects on the epithelial barrier in our explant culture model containing only resident colitogenic cells. To date, the effects of IFN- $\gamma$  and/or other proinflammatory cytokines on the intestinal epithelial barrier have been examined using reductionist models of human colonic cancer cell lines treated with high doses of rhIFN- $\gamma$  or in vivo mouse models of stress-induced IFN- $\gamma$  production involving recruited immune cells (42). Here we show that IFN- $\gamma$ , produced in situ by resident mucosal cells, is largely responsible for epithelial barrier damage, as shown by the significant – but not total – protection of crypt morphology (78%) when anti-IFN- $\gamma$  neutralizing antibodies were added to IL-10-depleted mucosa explant cultures. Whether this barrier disruption is due to IFN- $\gamma$  alone or in the context of an immune response (including low levels of TNF- $\alpha$ , which can potentiate the effect of IFN- $\gamma$ ) remains to be defined. Finally, IFN- $\gamma$  production creates a self-perpetuating loop of inflammation, as it leads to the destruction of a major source of intramucosal immunosuppression: the epithelial barrier.

IL-17 is responsible for chronic intestinal inflammation in IL-10 $^{-/-}$  mice (43) and a T cell-mediated colitis in mice (44). In our model, we noted a modest increase of IL-17 in IL-10-depleted mucosa explant cultures compared with IFN- $\gamma$  levels. These results, together with our demonstration that the epithelial lesions observed upon IL-10 depletion were strongly reduced by neutralizing anti-IFN- $\gamma$  antibodies, are in line with the suggestion of Elson et al. (44) that in experimental colitis, early disease is mediated by the Th1 subset (producing IFN- $\gamma$ ) and disease progression is caused by Th17 cells (producing IL-17).

TGF- $\beta$  is another important antiinflammatory cytokine, whose disruption in mice leads to multifocal inflammation including colitis (45, 46). Our experiments, based on the neutralization of TGF- $\beta$ RII, show that TGF- $\beta$  exerts a protective role in this explant culture model. Interestingly, adding TGF- $\beta$  to IL-10-depleted explant cultures did not modify the IFN- $\gamma$  response. This finding appears to suggest independent protective effects of TGF- $\beta$  and IL-10 in human colonic mucosa. However, we cannot rule out the hypothesis that IL-10-depleted mucosal immune cells became refractory to TGF- $\beta$  action, thus masking a possible connection between these 2 immunoregulatory cytokines, as previously reported (47, 48). Further work is needed to explore this issue.

We next sought to identify the primary trigger of IFN- $\gamma$  production and epithelial disruption in IL-10-depleted mucosa explant cultures. Products from commensal bacteria have been established as a possible trigger of the Th1-mediated immune response in IL-10 $^{-/-}$  mice (7). Here we showed that both LPS and commensal bacteria stimulated IFN- $\gamma$  production in IL-10-depleted mucosa. Indeed, in our model, despite the numerous washings, it was impossible to get rid of LPS from commensal bacteria present in the surgical resection specimens. This





**Figure 7**

Epithelial barrier apoptosis in IL-10–depleted mucosa explant cultures. Assessment of apoptosis in IL-10–depleted mucosa explant cultures (48 h), using M30 antibody, TUNEL method, or an antibody directed to activated caspase-3 (see Methods). Numerous apoptotic epithelial cells (brown) scored positive with M30 and activated caspase-3 antibodies, as well as with the TUNEL assay, in the remaining surface epithelium and in the crypts. Arrowheads indicate base of the damaged crypts. Original magnification,  $\times 400$ .

LPS had access to the whole mucosa explants. Because the LPS scavenger polymyxin B (49, 50) was able to prevent both IFN- $\gamma$  production and epithelial barrier damage, it is clear that LPS translocation to the mucosa was the trigger of the colitogenic response in IL-10–depleted mucosa explant cultures. This was likely achieved by activation of TLR4 receptors, since preliminary results showed that anti-TLR4/MD2 neutralizing antibodies were able to prevent IFN- $\gamma$  production (data not shown). However, we cannot completely exclude a synergism with other TLRs. In addition, we showed that LPS translocation in the control mucosa explants did not induce per se epithelial disruption and cytokine production. It is thus conceivable that IL-10, produced and secreted by resident cells of the intestinal mucosa including the intact epithelial barrier, prevents the deleterious effect of LPS translocation and that IL-10 plays an anergic role toward LPS. These findings have several important pathophysiological implications, because they predict that bacterial products may translocate through the intestinal epithelial barrier without triggering per se a pathological inflammation. Therefore, locally produced IL-10 exerts a critical role in maintaining mucosal homeostasis. In the inflamed gut, despite IL-10 expression by epithelial cells and the absence of correlation between IL-10 production and severity of disease (20), a relative deficiency in local mucosal bioavailability of IL-10 has been suggested based on the relocalization of IL-10–positive mononuclear cells into the submucosa compartment (20). Thus, the extent of IL-10 production by mucosal mononuclear cells may not be sufficient to control the production of proinflammatory cytokines at this site (20). Therefore, the local delivery of IL-10 is a promising approach for

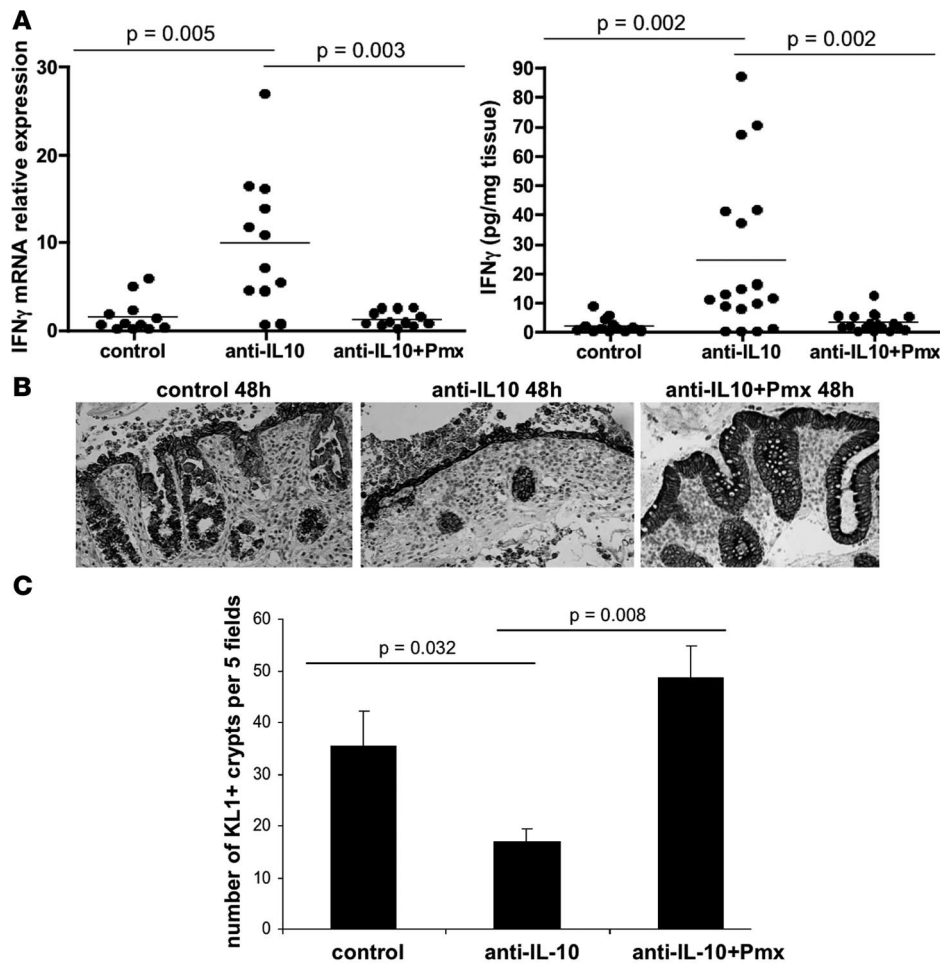
treating intestinal inflammation. The proof of principle comes from experiments in mice. In a model of Fabpi/IL-10 transgenic mice expressing IL-10 in the intestinal epithelium, colitis induced by dextran sulfate sodium administration or mediated by transfer of CD4<sup>+</sup>CD45RB<sup>hi</sup> splenocytes was greatly reduced (51). In addition, administration of adenoviral vectors encoding IL-10 that target the gastrointestinal tract, either in IL-10<sup>-/-</sup> mice or in chemically induced colitis, prevents and treats colitis (52, 53). Local delivery of IL-10 can also be achieved experimentally by administration of genetically engineered IL-10–secreting *Lactobacillus lactis* (54) or by stimulating local IL-10 production by probiotics (55). Our findings, together with the preliminary studies of Schreiber et al. showing that topical application of IL-10 by enema in patients with inflammatory bowel disease led to downregulation of proinflammatory cytokine production in vitro (56), should encourage further studies aimed at translating these experiments of local delivery to humans.

In conclusion, our findings establish that IL-10, mainly produced at the interface between the luminal content and the internal milieu, regulates a complex ecosystem composed of bacteria, the intestinal epithelial barrier, and resident colitogenic cells. In addition, this study shows that the important immunoregulatory cytokines IL-10 and TGF- $\beta$  play crucial roles in maintaining human colonic mucosa homeostasis.

## Methods

**Explant cultures.** Colon specimens were obtained from 34 patients undergoing surgery for colon carcinoma (21 men and 13 women; mean age, 64 years; range, 30–88 years). Data included in this report concern patients who did not undergo radiotherapy or chemotherapy. Fragments of the human colon, proximal ( $n = 16$ ) or distal ( $n = 18$ ), were taken at about 10 cm downstream to the tumor. The tissue fragments were processed according to the French guidelines for research on human tissues (57). Informed patient consent was obtained, according to the French bioethics law (58), beginning August 6, 2004. Immediately after removal, the tissues were placed in 4°C oxygenated Krebs solution, and the mucosa was carefully stripped from the underlying compartment made of muscularis mucosae and submucosa as previously described (59, 60). Fragments of 20–30 mg were cut out and pinned in Sylgard-coated Petri dishes and maintained in culture for 24 or 48 h in 2 ml RPMI 1640 medium (Invitrogen) containing 0.01% BSA and antibiotics (200  $\mu$ g/ml streptomycin, 200 U/ml penicillin, 1% fungizone; Invitrogen). The explants were maintained at 37°C in a 95% oxygen, 5% carbon dioxide humid atmosphere on a rocking platform at low speed. Mucosa explant cultures were performed in the absence or presence of neutralizing anti-IL-10 antibodies (monoclonal IgG1, 1  $\mu$ g/ml; Diaclone). Controls were performed with the isotype IgG1 control (mouse IgG1, 1  $\mu$ g/ml; Diaclone). In each experiment, at least 3 fragments were cultured for each condition. At the end of the 24- or 48-h cultures, the supernatants were centrifuged, and aliquots were stored at  $-80^{\circ}\text{C}$  for further analysis. Tissue specimens were cut into 2 fragments:





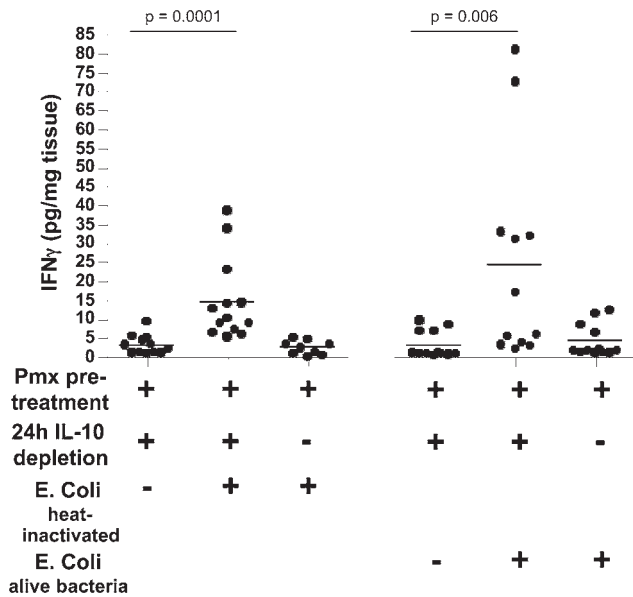
**Figure 8**

Involvement of bacterial LPS in the production of IFN- $\gamma$  and epithelial barrier disruption in IL-10-depleted mucosa explant cultures. Mucosa explant cultures were incubated for 48 h with or without anti-IL-10 antibodies and the LPS scavenger polymyxin B (Pmx, 10  $\mu$ g/ml). **(A)** IFN- $\gamma$  mRNA levels were measured by real-time PCR (left); IFN- $\gamma$  secretion was measured by ELISA (right). In IL-10-depleted explant cultures, polymyxin B abolished IFN- $\gamma$  upregulation, at both mRNA and protein levels. Horizontal lines represent mean values of 4 (left) or 5 (right) different experiments. **(B)** Immunoperoxidase staining of colonic epithelial cells with the anti-cytokeratin antibody KL-1 on paraffin sections. Polymyxin B prevented epithelial barrier disruption in IL-10-depleted mucosa explants. Original magnification,  $\times 200$ . **(C)** KL-1-positive intact crypt counts in 48-h mucosa explant cultures treated or not with anti-IL-10 antibodies and polymyxin B. Values are mean  $\pm$  SEM of 5 different experiments.

the largest was used for RNA extraction and real-time RT-PCR analysis, and the smaller fragment was used for histological examination after formalin fixation and paraffin embedding. Tissue integrity and cell viability of the explants were assessed by standard morphological analysis on H&E staining on paraffin sections. In some experiments aimed at blocking secreted IFN- $\gamma$ , anti-IFN- $\gamma$  neutralizing antibodies (monoclonal IgG1, 1  $\mu$ g/ml; Diaclone) were added to the culture medium, together with anti-IL-10 antibodies, for 48 h. In some experiments aimed at blocking the biological activity of TGF- $\beta$ s, mucosa explant cultures were incubated with anti-TGF- $\beta$ RII neutralizing antibodies (polyclonal goat antibody, 5  $\mu$ g/ml; R&D Systems) or with normal goat serum (control cultures). Some experiments were performed in the presence of polymyxin B (Sigma-Aldrich), a neutralizing agent of bacterial LPS (49, 50). In such experiments, dissected fragments were subjected to 3 10-min washes with the culture medium containing 30  $\mu$ g/ml polymyxin B on a rocking platform. The medium was subsequently changed, and the 24- or 48-h incubation was performed in the presence of 10  $\mu$ g/ml polymyxin B.

*Immunofluorescence and immunoperoxidase studies.* For IL-10 immunostaining followed by confocal microscopy, cryostat sections of human normal colon were performed, fixed in paraformaldehyde, and processed for immunofluorescence as reported previously (60). Briefly, tissue sections were incubated for 1 h with the anti-IL-10 monoclonal antibody (diluted 1:100; Diaclone) and then for 30 min with Alexa Fluor 488-conjugated goat anti-mouse antibodies (diluted 1:200; Invitrogen). Nuclear staining was performed with TOPRO-3 (1  $\mu$ M; Invitrogen). Sections were then mounted using Prolong antifade medium (Invitrogen). Imaging was performed on a Leica TCS-SP confocal laser-scanning microscope (Leica) equipped with an argon-krypton laser. Negative controls, performed by omitting the primary antibody or with isotype IgG1 controls, were used to set the parameters of photomultipliers. Sections were visualized with a  $\times 63/1.4$  oil objective lens, and image processing was performed using TCS-NT software (Leica).

For assessing tissue damage and counting crypts, we used on paraffin sections an immunohistochemical marker of epithelial cells, the pan-cytokeratin antibody KL-1 (diluted 1:50; Immunotech). Counts of



**Figure 9**

Effect of a commensal bacteria strain on IFN- $\gamma$  production by IL-10 depleted mucosa explant cultures. Mucosa explants were first washed with polymyxin B (30  $\mu$ g/ml) to neutralize endogenous LPS and then put into culture for 24 h in the presence or not of anti-IL-10 antibodies (1  $\mu$ g/ml) and in the presence or not of the commensal bacteria strain *E. coli* K12 MG1655 ( $10^7$  bacteria per explant), either heat-inactivated or live. IFN- $\gamma$  was measured in the culture supernatants by ELISA. Horizontal lines represent mean values of 3 different experiments.

viable crypts were performed independently by 2 observers on KL-1-stained paraffin sections. A minimum of 5 fields was counted at  $\times 200$  magnification. The results were expressed as the number of crypts per 5 fields. For assessing epithelial apoptosis, we used the following on paraffin sections: (a) the M30 monoclonal antibody (diluted 1:50; Roche Diagnostics), which recognizes a cytokeratin-18 neopeptide that becomes available at an early caspase cleavage event during apoptosis and is considered an early marker of apoptosis in epithelial cells (61, 62); (b) the TUNEL method, which detects apoptotic cells by labeling DNA strand breaks (Apoptag Peroxidase In Situ Apoptosis Detection Kit; Chemicon); and (c) the antibody against activated caspase-3 (diluted 1:100; Cell Signaling). An anti-BCL3 antibody was also used on paraffin sections (diluted 1:50; Santa Cruz Biotechnology Inc.). A standard streptavidin-biotin peroxidase method (LSAB kit; DakoCytomation) or the DakoCytomation ChemMate Envision detection kit (for caspase-3) were used according to the manufacturer's instructions. Negative controls were performed by omitting the primary antibody. DAB (3,3'-diaminobenzidine tetrahydrochloride) was used as a chromogen, and hematoxylin counterstaining was performed.

**Isolated human normal colonic epithelial cells.** Human colonic epithelial cells were isolated from histologically normal colon taken at a distance from the tumor of surgical resections for colon cancer, using a nonenzymatic dissociation technique as described previously (17). Preparations of colonocytes were devoid of contamination by immune cells (17).

**RNA extraction and real-time RT-PCR analysis.** Total RNA was extracted from (a) preparations of epithelial cells isolated from human normal colon and matched microdissected mucosa from 4 patients and (b) the 24- or 48-h mucosa explants from 20 patients, using the RNAeasy mini kit (Qiagen) and the Fast Prep cell disrupter (Bio 101). RNA was reverse transcribed as previously described (60).

Real-time PCR was performed as previously described (60) on a Rotor-gene 2000 instrument (Corbett Research), using primers for  $\beta$ -actin, TNF- $\alpha$  (60), IFN- $\gamma$  (5'-AGCTCTGCATCGTTTTGGGTT-3' and 5'-GTTC-CATTATCCGCTACATCTGA-3'), and IL-10 (5'-GATGCCTTCAGCA-GAGTGAA-3' and 5'-GCAACCCAGGTAACCCTTAAA-3'). PCR amplification was performed using Titanium Taq DNA polymerase (Clontech) as previously described (60). *BCL3* and TGF- $\beta$ 1 mRNA were quantified using commercially available kits (TaqMan Gene Expression Assays Hs00180403

and Hs00171257, respectively; Applied Biosystems), using a 7700 thermocycler (Applied Biosystems). For each sample, the ratio between the relative amount of each specific transcript and  $\beta$ -actin was calculated.

**Immunoblot analysis.** For total protein extraction, human normal colonic epithelial cells ( $n = 3$ ) and microdissected mucosa before culture ( $n = 3$ ) were lysed in RIPA buffer as previously described (63). Proteins (50  $\mu$ g) were separated by electrophoresis on 15% polyacrylamide gel and transferred onto nitrocellulose membranes (0.45  $\mu$ m porosity; Bio-Rad). After blocking, membranes were incubated with the monoclonal anti-IL-10 antibody (diluted 1:250; Diaclone) and then with a horseradish peroxidase-conjugated goat anti-mouse antibody (diluted 1:1,000; Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected on films using an enhanced chemiluminescence substrate (Roche Diagnostics) as previously described (63).

**Cytokine determination assay.** IL-10, IFN- $\gamma$ , TNF- $\alpha$ , and IL-17 released from tissue to the culture medium were determined by ELISA (IL-10 and IFN- $\gamma$  kits, Abcys; OptEIA Human TNF- $\alpha$ , BD Biosciences; IL-17, e-Bioscience) in samples collected at the end of the experiment, according to the manufacturers' protocols. Results are expressed as pg/mg wet tissue.

**Detection of bacterial endotoxin in the culture supernatants.** Bacterial endotoxins (LPS) were quantified using the chromogenic Limulus amoebocyte lysate (LAL) assay (C. River Endosafe) by the Institut Départemental d'Analyse et de Conseil (Nantes, France).

**Explant culture in the presence of a commensal bacteria strain.** The *E. coli* K12 MG1655 strain was used as a representative of commensal nonpathogenic *E. coli*, whose genome is entirely sequenced (64). *E. coli* MG1655 was aerobically grown overnight with shaking in BBL Brain Heart Infusion medium (BD) at 37°C to reach stationary growth phase, and the bacterial number was determined by serial dilutions and plating 100  $\mu$ l in duplicate on Mueller-Hinton 2 agar plates (BioMerieux). The bacteria, either live or heat inactivated (30 min treatment at 70°C), were diluted in the mucosa explant culture medium to obtain  $10^7$  bacteria per explant Petri dish (2 ml). Before a 24-h incubation with live or heat-inactivated *E. coli* MG1655, the mucosa explants were preincubated with polymyxin B (30  $\mu$ g/ml in culture medium; 3 times, each 15 min) to get rid of LPS from commensal bacteria present in surgical resections.

**Statistics.** Statistical analyses were performed with GraphPad Prism version 4.0 (GraphPad Software Inc.) using the Mann-Whitney *U* test to compare mRNA and protein levels between control and treated explant cultures. A *P* value less than 0.05 was considered significant.



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