COLONIC INFLAMMATION

Prevention of experimental colitis by parenteral administration of a pathogen-derived immunomodulatory molecule

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Background: Filamentous haemagglutinin (FHA) of *Bordetella pertussis* subverts host immune responses by inhibiting interleukin (IL)12 and enhancing IL10 production by macrophages and dendritic cells, and promoting the induction of regulatory T cells.

Hypothesis: Injection of FHA would ameliorate disease in a T cell-dependent model of colitis via the induction of anti-inflammatory cytokines and regulatory T cells.

Methods: Colitis was induced by injection of CD4CD45RB^{high} naive T cells into severe combined immunodeficient (SCID) mice. Mice were treated with four subcutaneous injections of FHA or buffer alone.

Results: Parenteral injection of FHA stimulated IL10 and/or transforming growth factor β production in local and mesenteric lymph nodes and Peyer's patches of mice 2–6 h after administration. Compared with phosphate-buffered saline-treated mice, FHA-treated SCID mice had significantly ($p < 0.01$) less weight loss, lower colon weight, less colon shrinkage and reduced inflammatory lesions. The therapeutic effect of FHA was associated with enhanced IL10 and reduced type 1 and type 2 T helper cytokine production by spleen cells. Finally, FHA also attenuated the symptoms of colitis in SCID mice transferred with CD4CD45RB^{high} T cells from IL10-deficient mice.

Conclusions: Our finding shows that FHA suppresses type 1 T helper and pro-inflammatory cytokines, and ameliorates disease activity in a chronic T cell-dependent model of colitis, an effect that was not dependent on IL10 production by T cells, but was associated with induction of anti-inflammatory cytokines in vivo. Having already been used as a pertussis vaccine component in children, FHA is a promising candidate for clinical testing in patients with Crohn's disease.

Chronic intestinal inflammation in Crohn's disease is associated with excessive production of type 1 T helper (Th1) and pro-inflammatory cytokines, interferon (IFN) γ , tumour necrosis factor (TNF) α and interleukin (IL)12.¹ Evidence proves that these harmful inflammatory and tissue-destructive immune responses may result from a defect in anti-inflammatory responses or loss of tolerance to commensal bacterial antigens. The production of anti-inflammatory cytokines, IL10 and transforming growth factor (TGF) β in response to Toll-like receptor (TLR)2 ligands and enteric bacteria is greatly reduced, and Th1 responses are enhanced in patients with Crohn's disease having a mutation in NOD2, an intracellular pathogen recognition receptor for muramyl dipeptide from bacterial peptidoglycan.^{2,3} Alternatively, immune-mediated diseases can arise through defective counter-regulation of inflammatory responses as a result of a failure to generate regulatory T cells.⁴ Transfer of regulatory T cells has been shown to confer protection against intestinal inflammation in animal model of colitis.⁵ Therefore, strategies that target the induction of anti-inflammatory cytokines and regulatory T cells in vivo have considerable promise in the development of new treatments for Crohn's disease.

Pathogenic bacteria use various evolutionary-adapted mechanisms to infect the host; these bacterial virulence factors are necessary to penetrate natural barriers and spread in the body, but are also crucial in avoiding or suppressing innate and adaptive immune responses, resulting in decreased clearance of the pathogen from the host. *Bordetella pertussis* is a Gram-negative bacterium that causes whooping cough, a severe and protracted respiratory disease in young children.⁶ Recovery from infection is associated with the development of *B pertussis*-specific Th1 cells.⁷ The protracted course of infection is due to several strategies of *B pertussis* to circumvent protective immune

responses. Filamentous haemagglutinin (FHA) is a major virulence factor of *B pertussis*, whose function is primarily adhesion, but is also involved in the immune subversion by bacteria. FHA stimulates IL10 and inhibits TLR-induced IL12 production by macrophages and dendritic cells, resulting in the development of IL10-secreting type 1 regulatory T cells.^{8,9} Furthermore, the inhibition of protective immune responses by regulatory T cells during acute infection with *B pertussis* seems to depend on the presence of a functional TLR4 receptor.¹⁰

We describe the therapeutic efficacy of parenteral administration of FHA for the treatment of experimental colitis. We hypothesised that FHA would induce anti-inflammatory cytokines from innate cells in vivo, which in turn would lead to the generation of regulatory T cells capable of controlling the induction of colitogenic effector T cells. Our findings show that systemic administration of FHA can modulate innate immune cells to produce IL10 and transforming growth factor (TGF) β , and these anti-inflammatory cytokines either directly or through the induction of regulatory T cells, can reduce intestinal inflammation in a T cell-mediated model of colitis.

MATERIALS AND METHODS

Induction of CD4CD45RB^{high} transfer colitis

The animal studies ethics committee of the University of Amsterdam, The Netherlands, and the Bioresources Ethics Committee of Trinity College, Dublin, Ireland approved all experiments. BALB/c mice (HSD NL or Harlan, UK), IL10-defective (IL10^{-/-}) BALB/c mice and severe combined

Abbreviations: FHA, filamentous haemagglutinin; IFN, interferon; IL10^{-/-}, interleukin-10-defective; PBS, phosphate-buffered-saline; Th1, type 1 T helper cell; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumour necrosis factor; SCID, severe combined immunodeficient

immunodeficient (SCID) mice on a BALB/c background (HSD NL) were housed under a filter top cage and supplied with autoclaved drinking water and food ad libitum. Chronic CD4CD45RB^{high} transfer colitis was induced as described previously.¹¹ CD4 T cells were enriched using a magnetic concentrating procedure; spleen cells were incubated with rat anti-mouse antibodies specific for B220 (clone RA3-6B2), Mac-1 (clone M1/70) and CD8a (clone 53-6.7; a gift from Dr R Mebius, Vrije Universiteit Medical Center, Amsterdam, The Netherlands) and then with anti-rat IgG-coated magnetic beads (Dyna Beads, Dynal, Hamburg, Germany). Beads were removed using a magnetic concentrator and washed twice, the CD4-enriched cell suspension contained 60% CD4 T cells as determined by fluorescence-activated cell sorter analysis. Enriched cells were resuspended again in RPMI 5% fetal calf serum and labelled with fluorochrome-conjugated antibodies: fluorescein isothiocyanate anti-mouse CD45RB and phycoerythrin-labelled anti-mouse CD4 (BD Biosciences, Mountain View, California, USA). Finally, cells were sorted in a CD4CD45RB^{high} and CD4CD45RB^{low} T cell fraction using a FACS Diva (B&D, Franklin Lakes, New Jersey, USA) and resuspended in sterile phosphate-buffered saline (PBS; ≥90% pure on reanalysis). SCID mice were injected intraperitoneally with 1×10^5 CD4CD45RB^{high} T cells for the induction of colitis; control mice were also injected with 2×10^5 CD4CD45RB^{low} T cells to prevent the development of colitis. Mice in the treatment groups received 10 µg of FHA in sterile PBS subcutaneously at days 0, 14, 28 and 42, and control mice received sterile PBS on the same days.

Assessment of inflammation

Body weights were recorded twice a week, and after termination of the experiment at day 56, the colon was harvested and opened longitudinally. After a rinse with 0.9% NaCl solution, to remove faecal material, colons were weighed, the length measured and prepared for routine histological examination with H&E or stained with hamster-anti-mouse CD3 (clone 145.2C11). An independent blinded pathologist (FWTK) scored the histological sections on a scale of 0–4, representing no inflammation to severe inflammation.¹¹

Cytokine production

For an assessment of in vivo cytokine production, BALB/c mice were injected subcutaneously with FHA or PBS, and 1–6 h later the Peyer's patches, mesenteric and inguinal lymph nodes were removed. The nodes were homogenised in PBS, centrifuged at 300 g for 10 min and supernatants removed for cytokine ELISA. IL10 and TGFβ protein concentrations were determined using cytokine-specific mouse DuoSet kits from R&D (Poole, Dorset, UK) as described by the manufacturers instructions. Pellets were resuspended in tetrazolium reduction inhibition reagent (Sigma) and RNA extracted by chloroform-isopropanol. After the normalisation of cDNA, TGFβ mRNA was determined by reverse transcriptase polymerase chain reaction. Primers used were sense 5'-AGA CCG AAT ACA GGG GCT TTC GAT TCA-3' and anti-sense 5'-CTT GGG CTT GCG ACC CAC GTA GTA-3'.

Cytokine production in treated and untreated SCID mice was determined using spleen cells and purified CD4 T cells. Spleen cell suspensions were prepared as described⁴ and suspended in complete medium, containing RPMI 1640 (Bio-Whittaker, Wokingham, Berkshire, UK) and 10% fetal calf serum (Bio-Whittaker). A total number of 1×10^5 cells were incubated in triplicate 96-well plates (Costar, Cambridge, Massachusetts, USA) in 200 µl complete medium and stimulated with FHA (5 µg/ml) or plate bound anti-CD3 (1:30 concentration; clone 145.2C11, gift from Dr R Mebius) and soluble anti-CD28 (1:1000 concentration; BD Biosciences). Cells were restimulated with CD3 and CD28, supernatants removed after 48 h, and IFNγ, TNFα, IL4 and IL5 production measured using a

cytometric bead array (BD Biosciences) or IL10 by two-site ELISA (R&D; detection limit 20 pg/ml).

Statistical analysis

Results are expressed as mean (SEM). Statistical significance of differences between mean values was assessed either by analysis of variance or where appropriate by Student's t test (two sample assuming equal variance). A single-factor analysis of variance was used to determine significance for changes in body weight during the course of the experiment. A p value <0.05, 95% confidence interval was used to indicate significance for each test.

RESULTS

FHA stimulates innate IL10 and TGFβ production in vivo

We examined whether parenteral administration of FHA activates the production of anti-inflammatory cytokines in vivo in local and intestinal-associated lymphoid tissue. BALB/c mice were injected subcutaneously with 10 µg FHA or PBS, mesenteric and inguinal lymph nodes, Peyer's patches and serum were removed after 1, 2 or 6 h and assessed for IL10 and TGFβ protein or mRNA (fig 1). Lymph node tissue had high constitutive expression of IL10. A significant increase in IL10 was detected in the serum, inguinal lymph nodes, mesenteric lymph nodes and Peyer's patches 2–6 h after subcutaneous injection of FHA. Parenteral injection of FHA also led to TGFβ production in vivo, with enhanced concentrations of TGFβ detected in inguinal and mesenteric lymph nodes. In the inguinal lymph nodes, enhancement of TGFβ protein was detected at 1 h and was further increased at 6 h. Moreover, TGFβ production induction was confirmed at the transcriptional level, with increases in TGFβ mRNA expression in FHA-treated mice 1 and 6 h after injection. FHA has previously been shown to stimulate IL10 production from dendritic cells and macrophages in vitro,^{8,9} and the rapid induction of IL10 and TGFβ observed here suggests that FHA can modulate innate immune cells to produce anti-inflammatory cytokines in vivo.

FHA attenuates T cell-mediated colitis

The CD4CD45RB^{high} transfer model of colitis is characterised by transmural infiltration of leucocytes in the colon, extensive epithelial cell hyperplasia, and the development of ulceration and crypt abscesses (fig 2A). The main compositions of infiltrating leucocytes are CD3 T cells (fig 2B). Moreover, the development of colitis can be prevented by coinjection of CD4CD45RB^{low} T cells containing a regulatory T cell population.¹² We have previously reported that FHA from *B pertussis* can stimulate IL10 production from dendritic cells and macrophages in vitro and can promote the development of regulatory T cells, which produce high amounts of IL10 and inhibit Th1 responses.⁹ Together with our current demonstration that FHA induces anti-inflammatory cytokines in vivo, we hypothesised that FHA might be able to confer protection against pathogenic immune responses in IBD and examined the potential protective efficacy of FHA in a model of chronic experimental colitis.

Three groups of mice were injected intraperitoneally with 1×10^5 CD4CD45RB^{high} T cells. One group of mice was treated with PBS subcutaneously every 2 weeks, one group of mice was treated with FHA subcutaneously every 2 weeks and the third group was injected intraperitoneally with 4×10^5 CD4CD45RB^{low} T cells on day 0. Body weights were recorded and mice that received PBS showed wasting disease 3 weeks after injection of CD4CD45RB^{high} T cells (fig 3A). By contrast, mice injected with FHA every 2 weeks showed significantly ($p < 0.01$) reduced weight loss compared with mice treated with PBS during the entire course of the experiment (fig 3A). Coinjection of CD4CD45RB^{low} with the CD4CD45RB^{high} T cells resulted in weight gain during the entire study period (fig 3A).

Figure 1 Filamentous haemagglutinin (FHA) stimulates innate anti-inflammatory cytokine production in vivo. BALB/c mice were injected subcutaneously with FHA (10 µg/mouse) or phosphate-buffered saline (PBS) and 1, 2 or 6 h later inguinal lymph nodes, mesenteric lymph nodes, Peyer's patches and serum were recovered. Interleukin (IL)10 protein concentrations were determined in serum (A). IL10 (B) and transforming growth factor (TGF)β (C) protein determined in homogenised lymphoid tissue by two-site ELISA. TGFβ mRNA was determined in inguinal lymph nodes by reverse transcriptase-polymerase chain reaction (D). Statistically significant differences compared with PBS-treated mice: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

At the end of the experiment, the colon weight and length were measured, both parameters indicative of the extent of colonic inflammation representing the formation of oedema, fibrosis and tissue infiltration of leucocytes. The significantly decreased weight loss seen in FHA-treated mice was reflected in a decreased colon weight and a normal colon length compared with PBS-treated mice (fig 3B; colon weight 140 (1) vs 343 (2) mg, $p < 0.01$; colon length 10.1 (0.6) vs 7.9 (0.3) cm, $p < 0.01$).

Colons were subsequently scored for different inflammatory characteristics on a scale from 0 to 4. After 8 weeks, SCID mice that received CD4CD45RB^{high} T cells and PBS-treated mice had marked epithelial cell hyperplasia, transmural leucocyte infiltration, ulceration and typical crypt abscesses (average histology score of 3 (1.26)). FHA-treated mice had a significantly ($p < 0.05$) decreased histology score compared with PBS-treated mice (average histology score 1.67 (0.52); fig 4). When

CD4CD45RB^{low} T cells were coinjected with CD4CD45RB^{high} T cells, colonic inflammation was confined to slight epithelial hyperplasia and only small numbers of leucocytes in the lamina propria (average histology score 0.83 (0.75), $p < 0.01$ vs PBS treated). In conclusion, these data show that subcutaneous injection of 10 µg FHA every 2 weeks is able to prevent the development of T cell-mediated colitis and wasting disease.

IL10 production is enhanced by FHA treatment, but T cell-derived IL10 is not necessary for the prevention of colitis

Having shown that FHA can prevent T cell-mediated colitis and given that FHA has been shown to induce IL10-secreting regulatory T cells,⁹ we examined the possible role of T cell-derived IL10 in the protective mechanism. Three groups of SCID mice were injected intraperitoneally with CD4CD45RB^{high} T cells from normal BALB/c mice; one group of mice received PBS subcutaneously every 2 weeks, one group received FHA subcutaneously every 2 weeks and the third group were injected intraperitoneally with 4×10^5 CD4CD45RB^{low} T cells on day 0. A fourth group of SCID mice were injected with 1×10^5 CD4CD45RB^{high} T cells from (IL10^{-/-}) mice and then treated with FHA every 2 weeks. In concordance with the first study, injection of CD4CD45RB^{high} T cells induced severe body weight loss up to 75% of the initial body weight (mean 90.5% (3.9%; fig 5A), which could be prevented by treatment with 10 µg FHA every 2 weeks (mean 104.8% (3.9%; fig 5A) or by coinjection with CD4CD45RB^{low} T cells (mean 112.8 (2%; fig 5A). Interestingly, FHA was equally effective in preventing weight loss in SCID mice injected with CD4CD45RB^{high} T cells from IL10^{-/-} or wild-type mice (mean 103.8 (3.2) vs 104.8 (3.9; fig 5A). In addition, PBS-treated SCID mice injected with CD4CD45RB^{high} T cells had significantly more colonic inflammation than FHA-treated SCID mice injected with wild-type or IL10^{-/-} CD4CD45RB^{high} T cells (3.3 (0.2) vs 1.7 (0.3) and 1.5 (0.2); fig 5B). Finally, CD4CD45RB^{high} transferred SCID mice coinjected with CD4CD45RB^{low} T cells significantly reduced inflammatory scores on histological assessment (3.3 (0.2) vs 1 (0.3); fig 5B). Therefore, FHA was equally effective in the prevention of severe colitis and wasting disease in SCID mice injected with CD4CD45RB^{high} T cells from wild-type and

Figure 2 Intestinal inflammation in an experimental T cell-mediated mouse model. Severe combined immunodeficiency mice were injected intraperitoneally with 1×10^5 CD4CD45RB^{high} T cells. Colons were removed after 56 days, and adjacent paraffin-wax-embedded sections were stained with H&E (A) or with antibodies specific for CD3ε (B).

Figure 3 Parenteral administration of filamentous haemagglutinin (FHA) prevents wasting disease in experimental colitis. Three groups of severe combined immunodeficient mice were injected intraperitoneally with 1×10^5 CD4⁺CD45RB^{high} T cells. One FFGHa prevents wasting disease in and experimental model colitis. Subcutaneous All group of mice were injected subcutaneously with phosphate-buffered saline (PBS) every 2 weeks, one group of mice was treated with 10 μ g FHA subcutaneously every 2 weeks and the third group of mice was injected intraperitoneally with 4×10^5 CD4CD45RB^{low} T cells on day 0. (A) Body weight was recorded twice weekly. Treatment with FHA or injection of CD4CD45RB^{low} T cells significantly decreased wasting disease induced by the injection of CD4CD45RB^{high} T cells ($p < 0.01$ and $p < 0.001$, respectively, using a single factor analysis of variance, 95% CI). (B) At the end of the experiment (day 56) the colon weights and colon lengths were measured. **Significant differences compared with PBS-treated mice ($p < 0.01$).

IL10^{-/-} mice, suggesting that T cell-derived IL10 was not essential for protection.

In an attempt to examine the modulatory effect of FHA on colitis in SCID mice transferred with CD4CD45RB^{high} T cells, we examined cytokine production by T cells stimulated via the T cell receptor, using anti-CD3 and anti-CD28 of spleen cells removed 8 weeks after induction of colitis in SCID mice injected with wild-type CD4CD45RB^{high} T cells treated with PBS, FHA or CD4CD45RB^{low} T cells or injected with CD4CD45RB^{high} T cells from IL10^{-/-} mice and treated with FHA. Spleen cells from SCID mice injected with CD4CD45RB^{high} T cells and treated with FHA produced significantly more IL10 than mice treated with PBS or coinjected with CD4⁺CD45RB^{low} T cells (244 (30) pg/ml vs 43 (7) and 48 (10) pg/ml; fig 6A,B). Moreover, IL10 production was significantly higher in unseparated spleen cells compared with purified CD4 T cells in FHA-treated mice (244 (30) vs 121 (31) pg/ml, fig 6A,B). As expected, no IL10 was detected when spleen cells

or CD4 T cells from SCID mice injected with IL10^{-/-} CD45RB^{high} T cells were stimulated with anti-CD3 and anti-CD28 (fig 6A,B).

Relatively high concentrations of the pro-inflammatory Th1-type cytokines, IFN γ and TNF α and lower levels of the Th2 cytokines IL4 and IL5 (fig 6C–F) were detected in spleen cells recovered from PBS-treated mice, which had severe colitis. By contrast, spleen cells of SCID mice treated with FHA and injected with naive T cells from wild-type or IL10^{-/-} mice produced significantly lower concentrations of IFN γ , IL4 and IL5, when compared with spleen cells from PBS-treated SCID mice (fig 6C,E,F). Suppression of Th1 and Th2 cytokines was also observed in CD4⁺CD45RB^{high} transferred SCID mice treated with FHA or injected with CD45RB^{low} T cells. These findings show that treatment of experimental colitis with FHA is associated with suppressed production of Th1 and Th2 cytokines detectable in the spleen, and enhanced production of the anti-inflammatory cytokines, IL10 and TGF β .

Figure 4 Histological evidence that filamentous haemagglutinin (FHA) can prevent colitis. Colitis was induced and mice were treated as described in the legend to fig 3. Colons were removed on day 56, and paraffin-wax-embedded sections were stained with H&E. Three representative histological sections are shown from severe combined immunodeficient mice injected intraperitoneally with 1×10^5 CD4CD45RB^{high} T cells and subsequently injected with phosphate-buffered saline (PBS) every 2 weeks from day 0 (A), 10 μ g FHA every 2 weeks from day 0 (B) or CD4CD45RB^{low} T cells at day 0 (C). A blinded pathologist assessed different inflammatory characteristics to determine the degree of colitis using a score from 0 to 4 (D). Significant differences compared with PBS-treated mice: * $p < 0.05$ and ** $p < 0.01$.

Figure 5 Role of T cell-derived interleukin (IL)10 in the protective effect of filamentous haemagglutinin (FHA). Severe combined immunodeficient mice were injected intraperitoneally with 1×10^5 CD4CD45RB^{high} T cells from wild-type or IL10^{-/-} BALB/c mice and treated every 2 weeks with phosphate-buffered saline (PBS) or FHA. Another group were injected with both CD4CD45RB^{high} and CD4CD45RB^{low} T cells. Body weight was recorded twice a week (A). At the end of the experiment (day 56) the colon was removed and prepared for histological examination and assessed for different inflammatory characteristics to determine the degree of colitis using a score from 0 to 4 (B). Significant differences compared with PBS-treated mice: * $p < 0.05$ and *** $p < 0.001$.

DISCUSSION

The important findings of this study are that parenteral administration of a pathogen-derived immunomodulatory molecule, FHA from *B. pertussis*, is capable of reducing the intestinal inflammation and other symptoms of colitis in a mouse model. Consistent with previous reports, SCID mice injected with CD4CD45RB^{high} T cells from naive BALB/c developed colitis, which was prevented by cotransfer of CD4CD45RB^{low} T cells.^{5, 13} Parenteral administration of FHA, which enhances IL10 production from macrophages and dendritic cells and promotes the induction of regulatory T cells,^{8, 9} prevented wasting disease and

the development of inflammatory lesions in the colon in SCID mice transferred with CD45RB^{high} cells. The attenuation of colitis in the CD45RB^{high} transferred SCID by treatment with FHA was associated with a reduction in pro-inflammatory Th1 cytokines and a concomitant increase in anti-inflammatory cytokine production by innate cells and T cells.

The CD4CD45RB^{high} naive T cell to SCID transfer model is a useful animal model for Crohn's disease, where Th1 cells play a key role in the pathogenesis of intestinal inflammation.¹⁴ Crohn's disease is triggered by environmental, immunological and genetic factors that cumulate in deficient regulatory

Figure 6 Filamentous haemagglutinin (FHA) treatment enhances interleukin (IL)10 and suppresses T helper (Th)1 and Th2 cytokine production. Spleen cells were recovered from the four groups of mice described in figure 5. Unseparated spleen cells (A) or purified splenic CD4 T cells (B) were stimulated in vitro with anti-CD3 and anti-CD28, and IL10 concentrations determined in supernatants by two-site ELISA. Interferon γ (C), tumour necrosis factor α (D), IL5, (E) and IL4 (F) concentrations were tested in supernatants of anti-CD3 and anti-CD28-stimulated spleen cells. Results are mean (SD) for triplicate cultures. Significant differences compared with CD4CD45RB^{low}-treated mice: * $p < 0.05$ and compared with FHA-treated and with CD4CD45RB^{low}-treated mice and ** $p < 0.05$.

immune responses (tolerance) against the normal commensal flora, the subsequent uncontrolled pro-inflammatory cytokine and Th1 responses lead to chronic transmural inflammation.¹ The exact mechanism as to how these tolerogenic immune responses fail is yet to be determined; however, it is likely to involve recognition of conserved microbial patterns by pattern recognition receptors, expressed on cells of the innate immune system, including dendritic cells and macrophages. Indeed, activated mature dendritic cells are present in resection specimens of patients with Crohn's disease.¹⁵

Powerful immunosuppressive therapies are used for the treatment of Crohn's disease, including TNF α antibodies and azathioprine. To our knowledge, these therapies mediate their effects through direct inhibition of a key pro-inflammatory mediator or the induction of apoptosis of activated T cells and not through modulation of dendritic cell function.^{16,17} Direct administration of the anti-inflammatory cytokine IL10 has also been explored, but IL10 has a short half-life in vivo¹⁸ and clinical trials showed only modest therapeutic benefit.¹⁸ Bacteria genetically engineered to express IL10 have also been shown to reduce colitis in animal models and in humans.^{19,20} An alternative approach adopted in this study was to stimulate production of anti-inflammatory cytokines in vivo, by targeting appropriate host cells with pathogen-derived immunomodulatory molecules.

The association between deficient pattern recognition signalling and Crohn's disease suggests that modulating dendritic cell function can be an alternative approach to the treatment of Crohn's disease.²¹ Dendritic cells can be manipulated to promote differentiation of naive T cells into regulatory T cells⁴ and depletion of natural regulatory T cells results in colitis and other autoimmune diseases.²² Although the antigenic target of pathogenic Th1 cells and potential protective regulatory T cells in colitis have not been identified, these cells may be triggered by commensal bacteria. Furthermore, there is circumstantial evidence that reduced incidence of infections, especially with helminth parasites, may explain the lower levels of autoimmune diseases in developed countries²³ and this may be related to the induction of regulatory T cells. Indeed, it has been reported that infection with *Heligmosomides polygyrus* enhanced Foxp3 expression in T cells and suppressed intestinal inflammation in IL10^{-/-} mice.²⁴

Like many pathogens, *B pertussis* produces a number of virulence factors, including FHA, which cause immune suppression in the respiratory tract of the infected host.⁶ FHA binds to the pattern recognition receptors, CD11b/CD18, primarily expressed on dendritic cells and macrophages, resulting in enhanced IL10 and suppressed IL12 production, and suppressed Th1 responses.^{9,25} Furthermore, FHA promotes the induction of regulatory T cells specific for bystander antigens in vitro⁹ and in vivo (Keogh, McGuirk and Mills unpublished data). Therefore, FHA has the capacity to modulate innate immune cell function, stimulating anti-inflammatory cytokines, which in turn may be amplified through the secondary induction of regulatory T cells. In the present study, injection of FHA led to an immediate (2–6 h) induction of IL10 and TGF β in regional and distant lymph nodes and Peyer's patches, suggesting that FHA modulates tissue dendritic cells or macrophages to migrate to lymph nodes and secondary lymphoid tissues, and to initiate anti-inflammatory responses. Both IL10 and TGF β are crucial in the prevention of T cell-mediated colitis^{5,13} and are likely to be responsible for the presence of IL10-producing T cells detected in the spleen of FHA-treated SCID mice. The recent demonstration that patients with Crohn's disease having NOD-2 mutations have defective IL10, and TGF β signalling in response to enteric bacteria³ suggests that activation of these anti-inflammatory cytokines by alternative signalling pathways may

reverse the defect in certain patients with Crohn's disease. Interestingly, FHA induced the same levels of protection against colitis in SCID mice transferred with IL10^{-/-} and wild-type CD4CD45RB^{high} T cells, suggesting that protection was not dependent on IL10 production by T cells that had differentiated from the transferred CD4CD45RB^{high} cells. However, this does not rule out the possibility that the progeny of CD4CD45RB^{high} cells may have differentiated into regulatory T cells that suppress immune responses through TGF β production or through cell-to-cell contact.²⁶ Finally, increased IL10 production in the mesenteric lymph nodes and Peyer's patches and by ex vivo splenic T cells indicate that FHA-stimulated immunomodulatory responses are not limited to local tissues but are also manifested at distant sites, including the intestine.

In conclusion, we have shown for the first time that a compound isolated from a potential pathogenic bacterium can be effective in the treatment of colitis and wasting disease that is characterised by an uncontrolled Th1 immune response. FHA suppresses pro-inflammatory cytokines, enhances anti-inflammatory cytokine production by cells of the innate immune system and generates IL10-producing T cells that in turn are not dependent on their IL10-producing capacity to suppress the development of colitis. Therefore, activation of regulatory dendritic cells, and, as a consequence induction of regulatory T cells alone or collectively may provide useful strategies for the prevention of colitis. FHA already has a proven record of safety in children, as an antigenic component of most current acellular pertussis vaccines.^{27,28} Having demonstrated that it can modulate dendritic cell function and prevent the generation of colitogenic T cells in the CD4CD45RB^{high} T cell transfer model of colitis, FHA has potential for clinical assessment in the induction of clinical response and remission in patients with Crohn's disease.

Competing interests: Declared (the declaration can be viewed on the Gut website at <http://www.gutjnl.com/supplemental>).

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REFERENCES

1. **Bouma G**, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003;**3**:521–33.
2. **Watanabe T**, Kitani A, Murray PJ, et al. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* 2004;**5**:800–8.
3. **Netea MG**, Kullberg BJ, de Jong DJ, et al. NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur J Immunol* 2004;**34**:2052–9.
4. **Mills KH**, McGuirk P. Antigen-specific regulatory T cells—their induction and role in infection. *Semin Immunol* 2004;**16**:107–17.
5. **Asseman C**, Mauze S, Leach MW, et al. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999;**190**:995–1004.
6. **Mills KH**. Immunity to *Bordetella pertussis*. *Microbes Infect* 2001;**3**:655–77.

- 7 **Ryan M**, Murphy G, Gohefors L, *et al*. *Bordetella pertussis* respiratory infection in children is associated with preferential activation of type 1 T helper cells. *J Infect Dis* 1997;**175**:1246–50.
- 8 **McGuirk P**, Johnson PA, Ryan EJ, *et al*. Filamentous hemagglutinin and pertussis toxin from *Bordetella pertussis* modulate immune responses to unrelated antigens. *J Infect Dis* 2000;**182**:1286–9.
- 9 **McGuirk P**, McCann C, Mills KH. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. *J Exp Med* 2002;**195**:221–31.
- 10 **Higgins SC**, Lavelle EC, McCann C, *et al*. Toll-like receptor 4-mediated innate IL-10 activates antigen-specific regulatory T cells and confers resistance to *Bordetella pertussis* by inhibiting inflammatory pathology. *J Immunol* 2003;**171**:3119–27.
- 11 **Van Montfrans C**, Rodriguez Pena MS, Pronk I, *et al*. Prevention of colitis by interleukin 10-transduced T lymphocytes in the SCID mice transfer model. *Gastroenterology* 2002;**123**:1865–76.
- 12 **Asseman C**, Read S, Powrie F. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4+ regulatory T cells and IL-10. *J Immunol* 2003;**171**:971–8.
- 13 **Powrie F**, Carlino J, Leach MW, *et al*. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* 1996;**183**:2669–74.
- 14 **Strober W**, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002;**20**:495–549.
- 15 **te Velde AA**, van Kooyk Y, Braat H, *et al*. Increased expression of DC-SIGN+IL-12+IL-18+ and CD83+IL-12-IL-18- dendritic cell populations in the colonic mucosa of patients with Crohn's disease. *Eur J Immunol* 2003;**33**:143–51.
- 16 **Van den Brande JM**, Braat H, van den Brink GR, *et al*. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 2003;**124**:1774–85.
- 17 **Tiede I**, Fritz G, Strand S, *et al*. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J Clin Invest* 2003;**111**:1133–45.
- 18 **van Deventer SJ**, Elson CO, Fedorak RN. Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. *Gastroenterology* 1997;**113**:383–9.
- 19 **Steidler L**, Hans W, Schotte L, *et al*. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* 2000;**289**:1352–5.
- 20 **Braat H**, Rotfiers P, Hommes DW, *et al*. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol Hepatol* 2006;**6**:754–9.
- 21 **Stagg AJ**, Hart AL, Knight SC, *et al*. The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria. *Gut* 2003;**52**:1522–9.
- 22 **Sakaguchi S**, Sakaguchi N, Asano M, *et al*. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;**155**:1151–64.
- 23 **Yazdanbakhsh M**, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002;**296**:490–4.
- 24 **Elliott DE**, Setiawan T, Metwali A, *et al*. Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice. *Eur J Immunol* 2004;**34**:2690.
- 25 **McGuirk P**, Mills KH. Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous hemagglutinin from *Bordetella pertussis*. *Eur J Immunol* 2000;**30**:415–22.
- 26 **Read S**, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 2000;**192**:295–302.
- 27 **Simondon F**, Preziosi MP, Yam A, *et al*. A randomized double-blind trial comparing a two-component acellular to a whole-cell pertussis vaccine in Senegal. *Vaccine* 1997;**15**:1606–12.
- 28 **Greco D**, Salmaso S, Mastrantonio P, *et al*. A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. Progetto Pertosse Working Group. *N Engl J Med* 1996;**334**:341–8.

EDITOR'S QUIZ:

Answer From question on page 342

The colonoscopy showed multiple ulcerations separated by normal mucosa involving the ileocaecal valve (fig 1) and ascending colon (fig 2). These endoscopic lesions may be seen in Crohn's disease, tuberculosis, amyloidosis, infectious disease, etc.

Histological examination showed amyloid deposits with Congo red staining (fig 3), and characterised by apple-green colour when viewed with a polarised microscope (fig 4). Intestinal amyloidosis was diagnosed. A blood test to search mutations on the gene causing familial Mediterranean fever (MEFV) detected a homozygote mutation in the M694V, confirming the diagnosis of gastrointestinal amyloidosis secondary to familial Mediterranean fever (FMF).

FMF is a hereditary episodic febrile syndrome. The disease is characterised by acute episodes of pain, usually in the abdomen, chest, joints, skin and muscles, accompanied by fever. FMF is an ethnically restricted disease, with autosomal recessive inheritance. It is more common in Sephardic Jews, Armenians, Turks, Arabs and Druze. The gene causing FMF (MEFV) was cloned from the short arm of chromosome 16. Five mutations (M694V, V726A, M694I, M680I, and E148Q) account for most MEFV changes.¹ FMF amyloidosis affects many organs, including the kidneys, adrenals, intestine, spleen, liver, stomach, thyroid gland, heart and lungs.² Most studies indicate that M694V homozygotes have an earlier age of onset and a higher frequency of arthritis, rash and amyloidosis. Amyloidosis of the gastrointestinal tract becomes clinically overt only in some patients. It may cause constipation, intractable diarrhoea and severe malabsorption. Ischaemic colitis and perforation of the large intestine has been reported.³ The diagnosis of systemic amyloidosis relies on tissue biopsy. Amyloid deposits are characterised by apple-green colour when stained with Congo red and viewed with a polarised microscope. Subtyping to AA or AL is usually aided using immunohistochemistry. The treatment of choice for FMF is daily oral colchicine⁴ which decreases the frequency and intensity of episodes and prevents the development of amyloidosis in compliant patients.⁵

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REFERENCES

- 1 **Touitou I**. The spectrum of familial Mediterranean fever mutations. *Eur J Hum Genet* 2001;**9**:473–83.
- 2 **Arutiunian VM**, Eganian GA, Khachatryan VA, *et al*. Selective involvement of the gastrointestinal tract in amyloidosis in a female patient with periodic disease and intact kidneys. *Ter Arkh* 1991;**63**:117–20.
- 3 **Kyser S**, Zevin D, Chagnac A, *et al*. Ischemic colitis complicating AA amyloidosis and familial Mediterranean fever. *Isr J Med Sci* 1993;**29**:212–14.
- 4 **Zemer D**, Pras M, Sohar E, *et al*. Colchicine in the prevention and treatment of the amyloidosis of familial Mediterranean fever. *N Engl J Med* 1986;**314**:1001–5.
- 5 **Livneh A**, Langevitz P. Diagnosis and treatment concerns in familial Mediterranean fever. *Baillieres Clin Rheumatol* 2000;**14**:477–98.