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(54) Title: USE OF A CYTOKINE–PRODUCING *LACTOCOCCUS* STRAIN TO TREAT COLITIS

(57) Abstract

The current invention relates to an administraton strategy for the delivery at the intestinal mucosa of cytokines or cytokine antagonists, preferably of acid sensitive anti–inflammatory agents, such as IL10 and/or soluble TNF receptor via the oral route. The prefered feature according to the invention is the inoculation with a suspension of recombinant *Lactococcus lactis* cells, which had been engineered to produce the respective proteins.

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Use of a cytokine-producing *Lactococcus* strain to treat colitis.

Summary of the invention

The invention generally relates to an administraton strategy for the delivery at the intestinal mucosa of cytokines, preferably of acid sensitive anti-inflammatory agents, such as IL10 and/or a soluble TNF receptor via the oral route. The prefered feature according to the invention is the inoculation with a suspension of live recombinant *Lactococcus lactis* cells, which had been engineered to produce the respective proteins. As example, mice were used in which a chronic inflammation of the distal colon had been induced by administration with dextran sulfate sodium (DSS). The treatment as scored by histological evaluation clearly resulted in a regression of the inflammation and disease symptoms. The finding is highly unexpected since, in order to exert activity at the colon following oral administration, the delivery system needs to pass the acidic environment of the stomach and the upper part of the small intestine respectively.

Background to the invention

The immune system in a mammal is diverse and complex and includes natural and adaptive immune mechanisms and reactions. The immune system is often described in terms of either humoral or cellular immune responses. Humoral immunity refers broadly to antibody production and actions by B-cells; cellular immunity is mediated by cells including T-cells, dendritic cells, neutrophiles, monocytes and macrophages. T-cells and B-cells are two categories of lymphocytes.

One of the mechanisms by which the immune system normally acts and regulates itself includes the production of so-called cytokines. It is known that cytokines mediate several positive and negative immune responses. Cytokines normally act by binding to a receptor on a target cell. The activity of cytokines

can be interfered with in several ways, for example by administration of soluble receptors (extracellular domains of the receptor) or by cytokine analogues or derivatives.

IL-10 is a cytokine capable of mediating a number of actions or effects. It is known that IL-10 is involved in controlling the immune responses of different classes or subsets of Th cells (T-helper cells).

Inflammatory bowel disease (IBD) refers to a group of gastrointestinal disorders characterized by a chronic non-specific inflammation of portions of the gastrointestinal tract. Ulcerative colitis (UC) and Crohn's Disease (CD) are the most prominent examples of IBD in humans. They are associated with many symptoms and complications, including growth retardation in children, rectal prolapse, blood in stools (e.g., melena and/or hematochezia), wasting, iron deficiency, and anemia, e.g. iron deficiency anemia and anemia of chronic disease or of chronic inflammation. The etiology or etiologies of IBD are unclear. Reference hereto is made in Wyngaarden and Smith (eds.) *Cecil's Textbook of Medicine* (W.B. Saunders Co. 1985), Berkow (ed.) *The Merck Manual of Diagnosis and Therapy* (Merck Sharp & Dohme Research Laboratories, 1982), and *Harrison's Principles of Internal Medicine*, 12th Ed., McGraw-Hill, Inc. (1991).

The incidence of IBD varies greatly with geographic location. A collaborative study over Europe shows an incidence per 100 000 of 10,4 for UC and of 5,6 for CD with 40% respectively 80% higher incidence for UC and CD in northern centres when compared to those in the south. As both UC and CD are long time affections, they correspond to real disturbances in the quality of life. Crohn's disease has a bimodal age distribution of onset showing striking peaks in incidence at 20 and 50 years of age. A higher incidence and more grievous disease profile is associated with the peak at younger age. This makes CD especially poignant as afflicted adolescents and young adults are virtually deprived form the high expectations form life, so particularly associated with this social group.

Ulcerative colitis refers to a chronic, non-specific, inflammatory, and ulcerative disease having manifestations primarily in the colonic mucosa. It is frequently

characterized by bloody diarrhea, abdominal cramps, blood and mucus in the stools, malaise, fever, anemia, anorexia, weight loss, leukocytosis, hypoalbuminemia, and an elevated erythrocyte sedimentation rate (ESR).

Complications can include hemorrhage, toxic colitis, toxic megacolon, occasional rectovaginal fistulas, and an increased risk for the development of colon cancer.

Ulcerative colitis is also associated with complications distant from the colon, such as arthritis, ankylosing spondylitis, sacroileitis, posterior uveitis, erythema nodosum, pyoderma gangrenosum, and episcleritis.

Treatment varies considerably with the severity and duration of the disease. For instance, fluid therapy to prevent dehydration and electrolyte imbalance is frequently indicated in a severe attack. Additionally, special dietary measures are sometimes useful. Medications include various corticosteroids, sulphasalazine and some of its derivatives, and possibly immunosuppressive drugs.

Crohn's Disease shares many features in common with ulcerative colitis. Crohn's Disease is distinguishable in that lesions tend to be sharply demarcated from adjacent normal bowel, in contrast to the lesions of ulcerative colitis which are fairly diffuse. Additionally, Crohn's Disease predominately afflicts the ileum (ileitis) and the ileum and colon (ileocolitis). In some cases, the colon alone is diseased (granulomatous colitis) and sometimes the entire small bowel is involved (jejunoileitis). In rare cases, the stomach, duodenum, or esophagus are involved. Lesions include a sarcoid-type epithelioid granuloma in roughly half of the clinical cases. Lesions of Crohn's Disease can be transmural including deep ulceration, edema, and fibrosis, which can lead to obstruction and fistula formation as well as abcess formation. This contrasts with ulcerative colitis which usually yields much shallower lesions, although occasionally the complications of fibrosis, obstruction, fistula formation, and abcesses are seen in ulcerative colitis as well.

Treatment is similar for both diseases and includes steroids, sulphasalazine and its derivatives, and immunosuppressive drugs such as cyclosporin A,

mercaptopurine and azathioprine. More recently developed treatments, some still in clinical trials, involve systemic administration (by injection) of TNF blocking compounds such as TNF-antibodies or soluble TNF receptor.

IBD represents a genuine problem in public health because of the absence of etiologic treatment. Although many patients are managed successfully with conventional medical therapy, such as anti-inflammatory corticosteroid treatment, most will have recurrent activity of disease and two-thirds will require surgery.

The cause of inflammatory bowel diseases is unknown. The pathogenesis of CD and UC probably involves interaction between genetic and environmental factors, such as bacterial agents, although no definite etiological agent has been identified so far. The main theory is that abnormal immune response, possibly driven by intestinal microflora, occurs in IBD. However, what is well established is that T-cells play an important role in the pathogenesis. Activated T-cells can produce both anti-inflammatory and pro-inflammatory cytokines. With this knowledge in hand, IBD can be counteracted in a rational manner. Novel anti-inflammatory therapies, which make use of neutralising monoclonal antibodies or anti-inflammatory cytokines, show the possibility to modulate the immune disregulations causative to IBD. A highly prominent and effective new therapy is systemic treatment with anti-TNF monoclonal antibodies as mentioned above. Single intravenous doses, ranging from 5 to 20 $mg.kg^{-1}$, of the cA2 infliximab antibody resulted in a drastic clinical improvement in active Crohn's disease. The use of systemically administered recombinant IL-10 in a 7 day by day treatment regime using doses ranging from 0.5 to 25 $\mu g.kg^{-1}$ showed reduced Crohn's disease activity scores and increased remission. A number of very promising therapies, either tangling pro-inflammatory cytokines or the establishment of T cell infiltrates, are currently emerging from experimental models. All these strategies however require systemic administration. The severe complications of IBD can be seriouly debilitating, and eventually may lead to death.

Detailed description of the invention

In US Patent 5,368,854, assigned to Schering Corp., a method is disclosed using IL-10 to treat inflammatory bowel diseases in mammals. In this method the cytokine is administered to a mammal having an IBD (inflammatory bowel disease). The administration of IL-10 as described in this reference is parenteral such as intravascular and preferably intravenous.

It is obvious however that such a route of administration for a (human) patient suffering from an IBD is not without drawbacks. A much easier and more convenient way is an oral administration of a medicament comprising a cytokine such as IL-10 or a cytokine-antagonist which has a similar therapeutic activity. More importantly, localized release of the therapeutic agent allows for higher efficacy and less unwanted side effects due to systemic activities.

In WO 97/14806, assigned to Cambridge University Technical Services Ltd., a method is disclosed for delivering biologically active polypeptides and/or antigens by using non-invasive bacteria, such as *Lactococcus*, by intranasal administration of said polypeptides in the body, especially at the mucosa.

However to treat an inflammatory bowel disease such as chronic colitis or Crohn's disease with a cytokine like IL-10, which is acid sensitive, is a very delicate and difficult task to accomplish. Therefore a system needs to be developed wherein the active compound (e.g. a cytokine or a soluble receptor) is delivered directly at the place where the compound is expected to exert its activity taken into account the problem of acid sensitivity of many cytokines, in particular of IL-10, and the requirement that after oral administration the delivery vehicle needs to pass the acidic environment of the stomach. Furthermore, various digestive enzymes degrade polypeptides as they pass through the stomach and the gut. Last but not least in-situ administration of the agent may allow to reach therapeutically effective concentrations which are difficult to achieve by more systemic routes of administration because of systemic toxicity or other limitations.

In order to achieve the recovery of a patient suffering from an IBD, it is necessary to restore the damaged cells and the organ comprising said damaged cells, for instance the colon.

The solution to the above described technical problem is achieved by providing the embodiments characterised in the claims.

It is our invention to use a cytokine-producing Gram-positive bacterial strain or a cytokine antagonist producing Gram-positive bacterial strain for the preparation of a medicament to treat inflammatory bowel disease.

Said cytokine or cytokine antagonist to be produced by the bacterial host strain is, for instance, IL-10, a soluble TNF receptor or a cytokine analogue such as the IL-12 derived p40 homodimer (an IL-12 antagonist), an Interferon-γ-antagonist, an IL-1 antagonist or a virus-coded cytokine analogue such as EBV BCRF1 (Baer et al., 1984), whereas the Gram-positive bacterial strain preferably is a *Lactococcus species* and more preferably a *Lactococcus lactis*.

Other Gram-positive bacterial strains to be used for the purpose of the current invention are *Bacillus subtilis*, *Streptococcus gordonii, Staphylococcus xylosus*, or a *Lactobacillus spec.* such as *Lactobacillus bulgaricus*, *Lactobacillus salivarius, Lactobacillus caseï, Lactobacillus helveticus, Lactobacillus delbrueckii* or *Lactobacillus plantarum.*

The inflammatory bowel diseases such as a chronic colitis, Crohn's disease or an ulcerative colitis can be treated according to the invention with an appropriate dosage of the active cytokine compound, preferably IL-10 or soluble TNF receptor, and provides unexpectedly a restoration of the diseased colon to an apparently normal and healthy state.

IL-10 can be administered alone or in combination with at least one additional therapeutic agent. Examples of such agents include corticosteroids, sulphasalazine, derivatives of sulphasalazine, immunosuppresive drugs such as cyclosporin A, mercaptopurine, azathioprine, and another cytokine. The co-administration can be sequential or simultaneous. Co-administration generally means that the multiple (two or more) therapeutics are present in the recipient during a specified time interval. Typically, if a second agent is administered within the half-life of the first agent, the two agents are considered co-

administered.

The invention disclosed herein thus concerns a localised delivery of IL-10 through in situ synthesis by recombinant *L. Lactis*. As a result thereof the inflammation is reduced by 50% in chronic colitis induced with DSS and prevents the onset of colitis in IL-10 -/- 129 Sv/Ev mice. So the method is equally efficient in comparison to powerful, well-established and accepted therapies relying on the systemic administration of anti-inflammatory proteins.

The vector used here, *L. lactis*, is a Gram positive food grade organism which is totally harmless. It is a non-colonising micro-organism. Accurate dosage and timing during treatment, shown here to be of great importance, can thus easily be obtained.

The critical requirement for viability of the vector is shown in the current invention. This indicates the need for in situ synthesis of IL-10. The vector is indeed capable to achieve this by showing de novo synthesis of IL-10 in the colon.

An efficient novel concept for protein based treatment in the intestinal tract is herewith disclosed. The treatment can be given by the oral route, which is farmost desirable for pharmacological formulations. It can exert effects up to the distal colon using a compound with intrinsic sensitivity for the route used. This method bypasses the need for systemic administration. It opens the possibility for the localised delivery of substances, which are unstable or difficult to produce in high quantities. It is intrinsically very cost effective.

This method may answer the question for sustained and localised presence of IL-10 in therapy at concentrations higher than desirable or even achievable through systemic delivery, with regard to latent side effects.

Some terms used in the current description are, for sake of clarity, explained hereafter.

Generally, the term "symptoms" refers to any subjective evidence of disease or of a patient's condition. This includes evidence as perceived by the patient. Examples of symptoms of IBD include diarrhea, abdominal pain, fever, melena, hematochezia, and weight loss.

The term "signs" refers generally to any objective evidence of a disease or condition, usually as perceived by an examining physician or features which would reveal themselves on a laboratory evaluation or other tests such as an ultrasonic study or a radiographic test. Some examples of signs of IBD include abdominal mass, glossitis, aphtous ulcer, anal fissure, perianal fistula, anemia, malabsorption, and iron deficiency. Occasionally, signs and symptoms overlap. For example, the patient complains of blood stools (a symptom), and a laboratory test of a stool sample is positive for blood (a sign).

The phrase "appropriate dosage" or "effective amount" means an amount or dosage sufficient to ameliorate a symptom or sign of an autoimmune condition or of an undesirable or inappropriate inflammatory or immune response. An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the method route and dose of administration and the severity of the side affects.

With "cytokine" is meant a polypeptide factor produced transiently by a range of cell types, acting usually locally, and activating the expression of specific genes by binding to cell surface receptors.

With "antagonist" is meant a compound that binds to but does not activate receptors, hence does inhibit the action of an agonist competitively.
"Agonists" are compounds that bind to and activate receptors (e.g., endogenous ligands such as hormones and neurotransmitters, chemically synthesized compounds, natural products like alkaloids).

Detailed description of the methods used in the current invention.

Culture media

GM17 is M17 (Difco, St. Louis) supplemented with 0.5 w/v % of glucose. GM17E is GM17 supplemented with 5µg/ml of erythromycin. BM9 contains per liter 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 2 mmol of $MgSO_4$, 25 mmol of $NaHCO_3$, 25 mmol of Na_2CO_3, 0.1 mmol of CaCl2, 5 g of glucose and 5 g of casitone (Difco). BM9E is BM9 supplemented with 5µg/ml of erythromycin.

Recombinant DNA techniques.

PCR amplification of DNA was performed with VENT polymerase and using conditions recommended by the manufacturer. DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out essentially as described (Sambrook et al., 1990). *L. lactis* was transformed by electroporation of cells grown in the presence of glycine (Wells et al., 1993).

Construction of the expression plasmids.

The plasmid pT1MIL10 (figure 1) was constructed by subcloning a PCR fragment, obtained with the primers (CAGTACAGCCGGGAAGACAAT and GCACTAGTTAGCTTTTCATTTTGAT) and performed on a cDNA clone containing mIL10 coding sequence. For the design of this strategy we made use of the mIL10 cDNA sequence as given in EMBL acc. nr. M37897. By utilization of the above mentioned primers, the mIL10 fragment could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX (figure 1), which is a pTREX1 derivative (Wells and Schofield in : Lactic Acid Bacteria: current advances in metabolism, genetics and applications. F. Bozoglu & R. Bibek, Eds., Nato ASI Series H, Vol.98, p. 37. Springer-Verlag, 1996.)

The plasmid pT1TR5AH (figure 1) was constructed by subcloning a PCR

fragment, obtained with the primers (CTGGTCCCTTCTCTTGGTGAC and CCACTAGTCTATTAATGATGATGATGATGATGCGCAGTACCTGAGTCCTGG GG) and performed on a cDNA clone containing sTNFr55 coding sequence. For the design of this strategy we made use of the TNFr55 cDNA sequence as given in EMBL acc. nr. L26349. By utilizing the above mentioned primers, the sTNFr 55 fragment was provided with a 6his tag at the 3'end and could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX.

Both plasmids code, downstream from the lactococcal P1 promotor, for fusion genes between the secretion leader from Usp45 (Van Asseldonk et al., Gene, 95, 155-160,1990) and mIL10 and sTNFr 55, respectively. Upon secretion, the leader sequence is cleaved off.

Identification of recombinant proteins

Recombinant mIL10 and msTNFr 55 could be observed in the supernatant of cultures of MG1363[pT1MIL10] and MG1363[pT1TR5AH], respectively (figure 2). For this test, 5 ml aliquots of the cultures were extracted with 2 ml phenol and the proteins were subsequently prepared from the organic phase by precipitation with 10 ml of ethanol. A part of the precipitate, equivalent to 1 ml of culture supernatant, was subjected to SDS-15% PAGE and immunoblotting. Culture samples were taken at relevant times in the growth phase of the bacteria, as described below.

The culture supernatant of MG1363[pT1MIL10] contained, on average, 1 $\mu g.ml^{-1}$ of murine IL10. Murine IL-10 activity of the supernatant was measured using a murine mast cell line MC/9 (Thompson-Snipes, L. et al., J. Exp. Med. 173, 507, 1991). Human IL-10 binds to murine IL-10R as was demonstrated by transfection experiments (Ho, A.S.Y et al., PNAS 90, 11267, 1993; Liu, Y. et al., J.Immunol. 152, 1821, 1994). 1 $U.ml^{-1}$ of IL-10 is defined as the amount of IL-10 that is able to inhibit 50% the level of IFN-gamma production of conA activated splenocytes (Fiorentino, D.F. et al., J.Exp.Med. 170, 2081, 1989). The ED50 for this effect is typically 0.3-0.6 $ng.ml^{-1}$. When measured along with a standard of known activity (Biosource International, CA) the MG1363[pT1MIL10] culture

supernatant revealed an activity of approximately 8000 $U.ml^{-1}$. Berg et al. (J. Clin. Invest 98, 1010-1020) report a specific activity of approximately 1.0×10^7 $U.mg^{-1}$ for recombinant mIL10. From these considerations and taking into account the variations in the method used, we concluded that the recombinant mIL10, present in the MG1363[pT1MIL10] culture supernatant, displayed full biological activity. No IL10 activity could be detected in the supernatant of the control cultures, MG1363 or MG1363[pTREX1].

The culture supernatant of strain MG1363[pT1TR5AH] contained, on average, 200 $ng.ml^{-1}$ msTNFr 55. Loetscher et al. (1991) showed that complete inhibition of TNF cytotoxic activity by sTNFr 55 was only obtained from a molar ratio of 1000 : 1 of sTNFr 55 to TNF and higher. The soluble recombinant TNFr 55 which had been recovered from the culture supernatant of MG1363(pT1TR5AH) showed an equal inhibitory effect on TNF as had been reported for the indigenous product. This was demonstrated by mixing up and thus competing out a titration series of TNF with a titration series of recombinant sTNFr and measuring TNF activity in a cytotoxicity assay as described (Espevik, T and Nissen-Meyer, 1986).

Pretreatment of the mice

For the induction of chronic colitis, mice were pre-treated as described by Kojouharoff et al. Clin Exp Immunol 107, 353, 1997. Six to eight weeks old female Balb/c mice received four cycles of treatment with DSS. Each cycle consisted of 5% DSS in the drinking water for 7 days, followed by a 10-day interval during which they received normal drinking water. Four to six weeks after completion of the last DSS cycle, mice were treated with the *L. lactis* strains as indicated.

Legends to the figures.

Figure 1 : Overview of the plasmids used

Figure 1 a : schematic maps of the plasmids used. P1 is the lactococcal P1 promotor as in Waterfield et al, (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococcal Usp45 (van Asseldonck et al, 1990), mil 10 is a DNA fragment encoding the mature part of murine interleukin 10, tr55 is a DNA fragment encoding the soluble part of type 1 TNF receptor, H6 is a fragment encoding 6 histidine residues, Em^r is the erythromycin selection marker.

Figure 1b : DNA sequences of pTREX1 and pT1NX

Figure 1c : DNA sequences of pTIMIL10 and pT1TR5AH

Figure 2 :

Protein profile following SDS-PAGE of the culture supernatant of the indicated strains after immunoblot, revealed with anti-murine interleukin 10 (panel A) or anti-murine type 1 TNF receptor and anti-6 His (panel B) antisera.

Figure 3 :

Average of colon length of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pT1TR5AH was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis strain* MG1363pT1MIL10 was orally administered.

Figure 4 :

Average of epithelial damage score in the distal colon of groups of mice in which : a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered, c) chronic colitis had been induced

with DSS and to which subsequently *L. lactis* strain MG1363pT1TR5AH was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pT1MIL10 was orally administered.

Figure 5 :

Average of inflamatory infiltrate score in the distal colon of groups of mice in which : a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis strain* MG1363pT1TR5AH was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pT1MIL10 was orally administered.

Figure 6:

Representative sections of mice distal colon stained with haematoxylin and eosin.

normal tissue : untreated animals

DSS colitis : animals pretreated with DSS to acquire chronic colitis

DSS colitis, MG1363pT1MIL10 treatment : animals pretreated with DSS to acquire chronic colitis to which subsequently *L. lactis* strain MG1363pT1MIL10 was orally administered. DSS colitis, MG1363pTREX1 treatment : animals pretreated with DSS to acquire chronic colitis to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered.

Figure 7:

Statistical evaluation of the histology. The colon sections were randomly numbered and interpreted blind. Scores from individual mice were subsequently decoded and the regrouped numbers were analysed statistically. The DSS colitis panel shows histological sumscores for the distal colon of blank mice and of mice induced with DSS to acquire chronic colitis, either untreated or treated with *L. lactis* cultures. The score is a sum of scores for epithelial damage and lymphoid infiltrate, both ranging between 0 and 4. Groups of mice (n = 10) were

alternatively treated with MG1363, MG1363(pTREX1) or MG1363(pT1MIL10) (= IL-10) for two (= 2w) or four (= 4w) weeks. Some of the cultures were irradiated with uv (= + uv) prior to inoculation, which reduced cell viability over 10^6 times. The IL-10-/- colitis panel shows histological sumscores of groups (n = 5) of 7 week old untreated, TREX treated and IL-10 treated female 129 Sv/Ev IL-10-/- mice. The histological score is a sum of the degree of inflammation in the proximal, middle and distal colon, all ranging between 0 and 4. Error bars represent s.e.m.

Figure 8:
Representation of bacterial viability after irradiation as measured at OD_{600}.

In order to further disclose and thus clarify the current invention some examples are given hereunder.

Examples

Example 1.

Treatment of the mice with live *L. lactis*

Storage of expression strains.
Freshly streaked cultures of the *L. lactis* expression strains were inoculated in 10 ml of GM17 or GM17E depending on the absence or presence of an expression plasmid and grown overnight at 30°C. The overnight cultures were diluted 1/100 in fresh GM17 or GM17E and pregrown for 3 hours at 30°C. The cells were harvested by centrifugation and resuspended in BGM9 or BGM9E, depending on the presence of plasmids. These cultures were grown for 5 hours at 30°C. The protein profile of these cultures was analysed by performing Western immunoblotting on an equivalent of 1 ml of culture supernatant using either antiserum directed towards sTNFr 55 or IL10 respectively. The protein profile showed the presence of sTNFr 55 and IL10 in the appropriate lanes

(figure 2). 5 ml of the original GM17 or GM17E overnight cultures was supplemented with 5 ml of glycerol and stored at –20°C. These stocks were used as starter material for several experiments. Protein analysis throughout a series of individual experiments showed that a high degree of reproducibility in the production of the recombinant proteins could be obtained by this procedure.

Weeks 1 and 2

Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. The cells were harvested by centrifugation and resuspended in 1 ml BM9 or BM9E. Control, healthy mice and mice with induced colitis were inoculated on a daily basis with 100 µl aliquots of these cell suspensions.

Weeks 3 and 4

Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. These cultures were diluted 1/25 in 10 ml of BM9 or BM9E and grown for 3 hours at 30°C. Aliquots of 200 µl were intragastrically (peroral) administered into mice on a daily basis.

Example 2.

Determination of histological score

Histological score was determined essentially as described by Kojouharoff et al. Clin Exp Immunol 107, 353, 1997.

Mice were killed by cervical dislocation. The colon was removed and washed with PBS. The distal third of the colon was cut longitudinally, laid on filter paper and fixed with 10% formalin in PBS overnight. Sections of the parafin-embedded material were made longitudinally. Three 3-µm sections were cut at an intermediate distance of 200 µm. The sections were stained with haematoxylin-eosin. Histological analysis was performed in blind fashion. Mice were scored individually, and each score represented the mean of three sections.

Histology was scored as follows:

Infiltration : 0, no infiltrate; 1, infiltrate around crypt bases; 2, Infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4 infiltration of the L. submucosa.

Epithelial damage: 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas and/or foci of polyploid regeneration.

Colonic length was measured immediately after dissection and placement on a paper towel.

The pathology of chronic colitis is, amongst other parameters, characterised by a decrease in length of the colon and by epithelial damage and infiltration of lymphocytes to a more or less substantial extent.

Figure 3 clearly shows an increase in colon length after the treatment of the inflamed mice with MG1363[pT1MIL10] and, although to a lesser extent, after the treatment of the mice with MG1363[pT1TR5AH].

Figure 4 and 5 show the onset of recovery from chronic colitis, in which mice treated with MG1363(pT1MIL10) appear to improve more extensively than those mice which had been treated with MG1363[pT1TR5AH].

Figure 4 shows the histological score of epithelial damage whereas figure 5 shows inflammatory infiltrate, both determined as described previously.

Figure 6 shows the histology of normal tissue, compared to inflamed and treated tissue.

In the normal histology one can observe a continuous array of crypts of equal length. In the crypts, numerous goblet cells can be observed. A low number of lymphocytes is present in the mucosa. No lymphocytes are present in the submucosa. In the inflamed tissue, one can see the disappearance of the organised crypt structures, ranging from differences in length to complete absence of structure. Also, in the relicts of the crypts no goblet cells are present. One can observe a large increase of the thickness of the mucosa due to a massive infiltration of lymphocytes. The lymphocytes tend to form

ulcerations. In severe cases, infiltration of lymphocytes can also be observed in the submucosa. The epithelium, however, remains intact. The negative control of treatment with MG1363(pTREX1) shows a pathology reminescent of that of heavilly inflamed tissue. Mice treated with MG1363 (pT1MIL10) show an almost complete restitution of the normal histology, revealing only slight remainders of infiltrating lymphocytes in the mucosa. Mice treated with MG1363[pT1TR5AH] show an intermediate degree in pathology.

Figure 7 shows the statistic evaluation of histological scores obtained from individual mice following treatment with the indicated *L. lactis* strains (group size = 10). The score was recorded after blind interpretation of slides from the distal colon as described (Kojouharoff et al.,1997). Each mouse was interpreted according to 3 longitudinal slides, equally spaced over the circumference of the colon. Both lymphoid infiltrate and epithelial damage were rated from 0 to 4 points and values for both parameters were summed for every mouse. Normal blank mice showed a histological score of 1 point. The mice induced for colitis are slightly over 5 points. All of the control groups for *L. lactis* treatment fluctuate around this number, with possibly a slightly higher tendency in some groups. The mice treated for 14 days with mIL-10 producing *L. lactis*, followed by 14 days of recovery however show an average of approximately 3 points. This is a decrease of nearly 50% in the pathology when measured against the difference between untreated and blank control groups. The reduction is significant ($p = 0.0151$).

Example 3

Due to the culture conditions used, a minor amount (40 ng) of mIL-10 is present in the supernatant of the inoculation suspension. To investigate whether this IL-10 brings about the observed reduction in the histological score we included treatment with UV killed IL-10 producer strains. These cultures were UV irradiated immediately prior to the inoculation. Figure 8 shows that irradiation reduced the bacterial viability to less than 1 in 10^6 cfu so that no further accumulation of IL-10 was observed. This was not associated with cell lysis

since no drop in OD_{600} was observed and no IL-10 precursor could be detected in the culture supernatant. The irradiation does not affect IL-10 bioactivity. Diseased mice treated for 2 or 4 weeks with the UV dispatched cultures show no difference in colon histology when compared to any of the control groups positive for enterocolitis. The fate of the residual IL-10 in the inoculation medium is most likely denaturation and breakdown in the stomach and duodenum. The acidity of the stomach, prior at pH 1,5, rises to pH6 immediately after inoculation. After 5 minutes a pH of 4 is reached, which further drops from 3,5 to 2,5 in the interval between 30 and 60 minutes after inoculation. IL-10 detected in the stomach 5 minutes after inoculation rapidly decreases in concentration and was only found in trace amounts in the duodenum at 30 minutes after inoculation. At later time-points no IL-10 was detected here nor in the jejunum or ileum.

Example 4

Seven serial inoculations of $3,4.10^9$ cfu of MG1363(pT1MIL10) were given to 129 Sv/Ev IL-10-/- mice, thereby respecting 1 hour intervals. The intestine was prepared out 30 minutes after the last inoculation and divided in the morphologic compartments. Immediately the tissues were homogenised in PBS with 1% BSA and 0,05% NaN_3. Cfu of MG1363(pT1MIL10) were determined as 7.10^6 in the stomach, $2,6.10^8$ in the duodenum, $2,8.10^7$ in the jejunum, 4.10^8 in the ileum, $8,4.10^8$ in the caecum and 7.10^8 in the colon. We have detected 70 ng of soluble IL-10 in the colon homogenate. None of the upstream compartments showed any IL-10 content. From this it is concluded that recombinant *L. lactis* can actively produce IL-10 in the colon.

Example 5

Prevention of enterocolitis in IL10-/- mice

The capacity of the approach described above was tested to prevent the onset of colitis in 129 Sv/Ev IL10-/- mice. These mice spontaneously develop a generalized enterocolitis in the frame between three and eight weeks of age (Kuhn et al.,Cell,1993;75:263-274). Inflammatory changes first appear in the cecum, ascending and transverse colon of 3-wk-old mutants. Progressive disease in ageing IL10-/- mice was characterised by an increased number of multifocal inflammatory cell infiltrates composed of mononuclear cells and neutrophils accompanied by moderate epithelial hyperplasia and slight mucin depletion from goblet cells. Small epithelial erosions and crypt abscesses were occasionally present and inflammation rarely involved the submucosa. IL10-/- mice used in our studies showed a less severe inflammation as described due to "clean" rather than "conventional" conditions of our animal facility.

When these mice are treated from week 3 on for 6 to 8 weeks with either anti IFN-γ or anti-IL-12 colitis can be prevented (Rennick et al., J-Leukoc-Biol.,1997 Apr; 61(4):389-396). We treated 3 weeks old mice by daily intra-gastric inoculation with IL-10 producing *L. lactis*. The mice were treated for 4 weeks with either mid-log or end-log cultures whilst an untreated group was kept under identical conditions. Figure 7 shows histological scores obtained as described (Berg et al., J-Clin-Invest;1996, Aug 15;98(4):1010-1020), with the exception that we did not examine the caecum. The non-treated mice show a mean histological score of approximately 4,5 points. This fits well with reported data, provided one takes into account the contribution of the caecal scores in these values and the slight age difference. The group of mice treated with MG1363(pT1MIL10) shows a mean histological score of 1,5 points which is only slightly over values reported for 3 week old mice (Berg et al., J-Clin-Invest;1996, Aug 15;98(4):1010-1020). As it is the sum of 3 values ranging from 0 to 4 points, this is considered as a very low score. From these data it is clear that the development of colitis can be prevented by this treatment.

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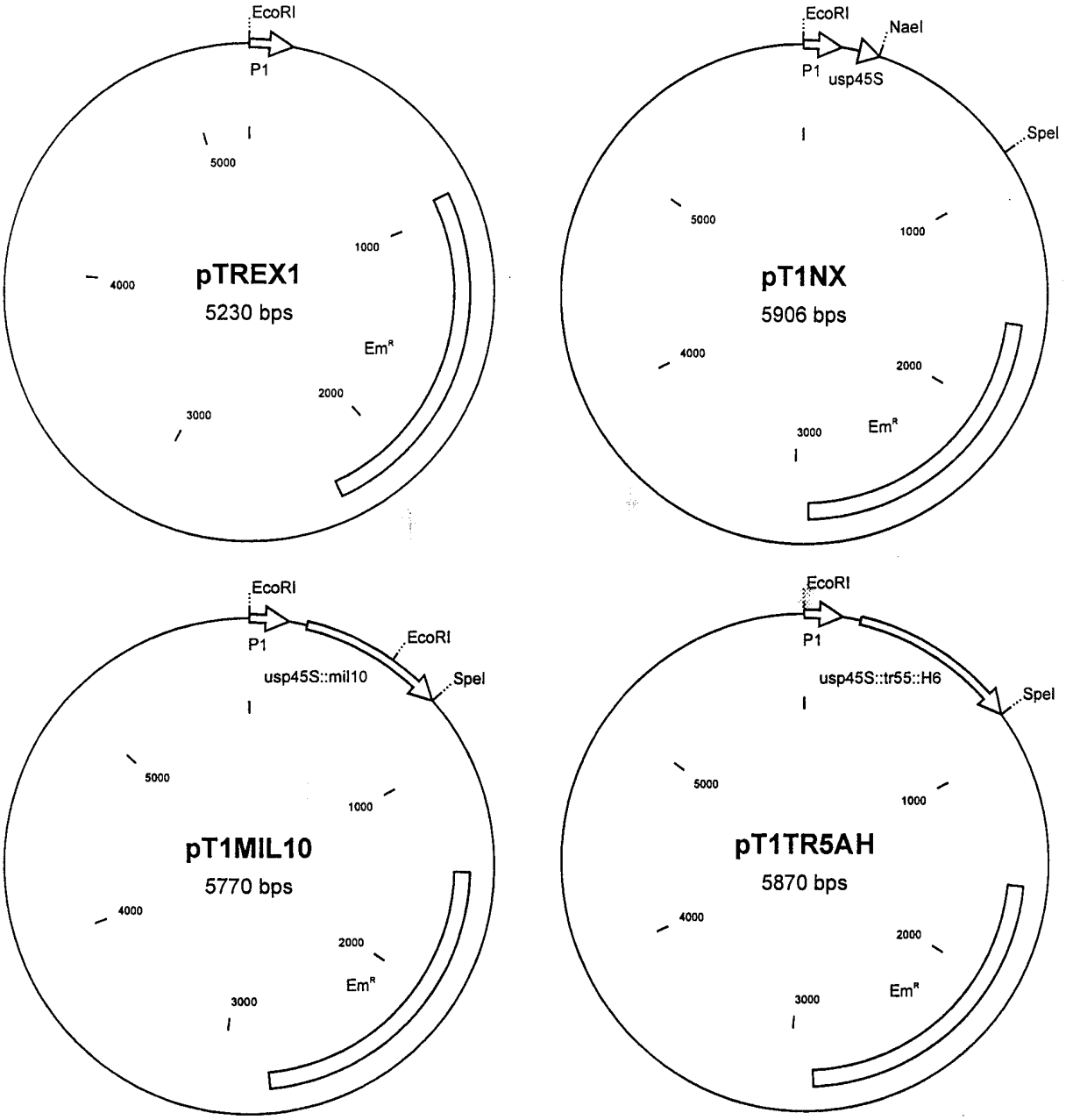
Claims

1. Use of a cytokine-producing Gram-positive bacterial strain or a cytokine antagonist-producing Gram-positive bacterial strain for the preparation of a medicament to treat inflammatory bowel disease.
2. Use of a Gram-positive bacterial strain according to claim 1 wherein the cytokine or cytokine antagonist is IL-10, a soluble TNF receptor or another TNF antagonist, an IL-12 antagonist, an Interferon-γ antagonist, an IL-1 antagonist or a virus-coded cytokine analogue such as EBV BCRF1.
3. Use of a Gram-positive bacterial strain according to claim 1 or 2 wherein the Gram-positive bacterial strain is a *Lactococcus species.*
4. Use of a Gram-positive bacterial strain according to claim 3 wherein the *Lactococcus species* is *Lactococcus lactis.*
5. Use of a Gram-positive bacterial strain according to claim 1 or 2 wherein the Gram-positive bacterial strain is *Bacillus subtilis*, *Streptococcus gordonii, Staphylococcus xylosus*, or a *Lactobacillus spec.*
6. Use of a Gram-positive bacterial strain according to any of the preceeding claims wherein the bowel disease is a chronic colitis, Crohn's disease or an ulcerative colitis.


EcoRI
P1
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1000
4000
pTREX1
5230 bps
Em^R
2000
3000
EcoRI
NaeI
P1
usp45S
SpeI
5000
1000
pT1NX
5906 bps
4000
2000
3000
Em^R
EcoRI
P1
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SpeI
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5770 bps
4000
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Em^R
3000
EcoRI
P1
usp45S::tr55::H6
SpeI
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1000
pT1TR5AH
5870 bps
4000
2000
Em^R
3000

Figure 1a

pTREX1 Figure 1B

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pTREX1 (cont.) Figure 1B (cont.)

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pT1NX Figure 1B (cont.)

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pT1NX (cont.) Figure 1B (cont.)

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pT1MIL10 Figure 1c

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pT1MIL10 (cont.) Figure 1c (cont.)

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pT1TR5AH Figure 1c (cont.)

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pT1TR5AH (cont.) Figure 1c (cont.)

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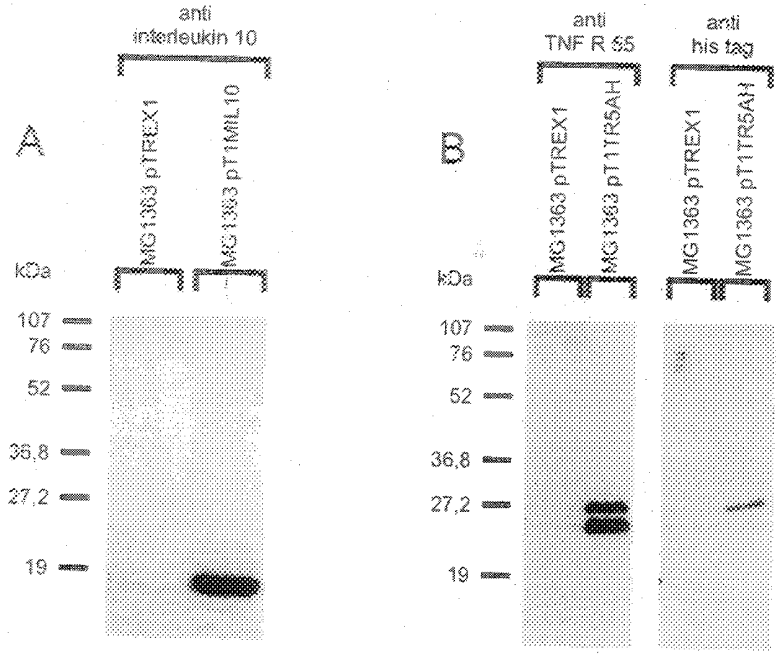
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anti
interleukin 10
MG1363 pTREX1
MG1363 pT1ML10
kDa
107
76
52
36,8
27,2
19
B
anti
TNF R 55
anti
his tag
MG1363 pTREX1
MG1363 pT1TR5AH
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107
76
52
36,8
27,2
19

Figure 2

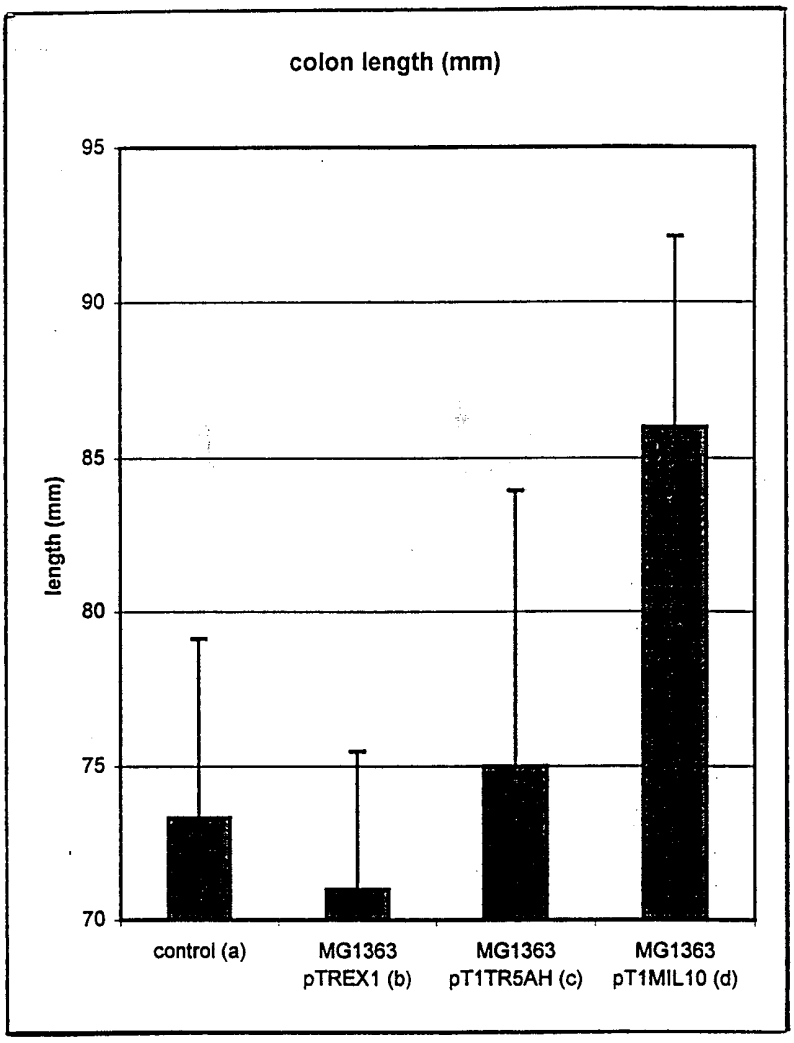

colon length (mm)
length (mm)
95
90
85
80
75
70
control (a)
MG1363 pTREX1 (b)
MG1363 pT1TR5AH (c)
MG1363 pT1MIL10 (d)

Figure 3

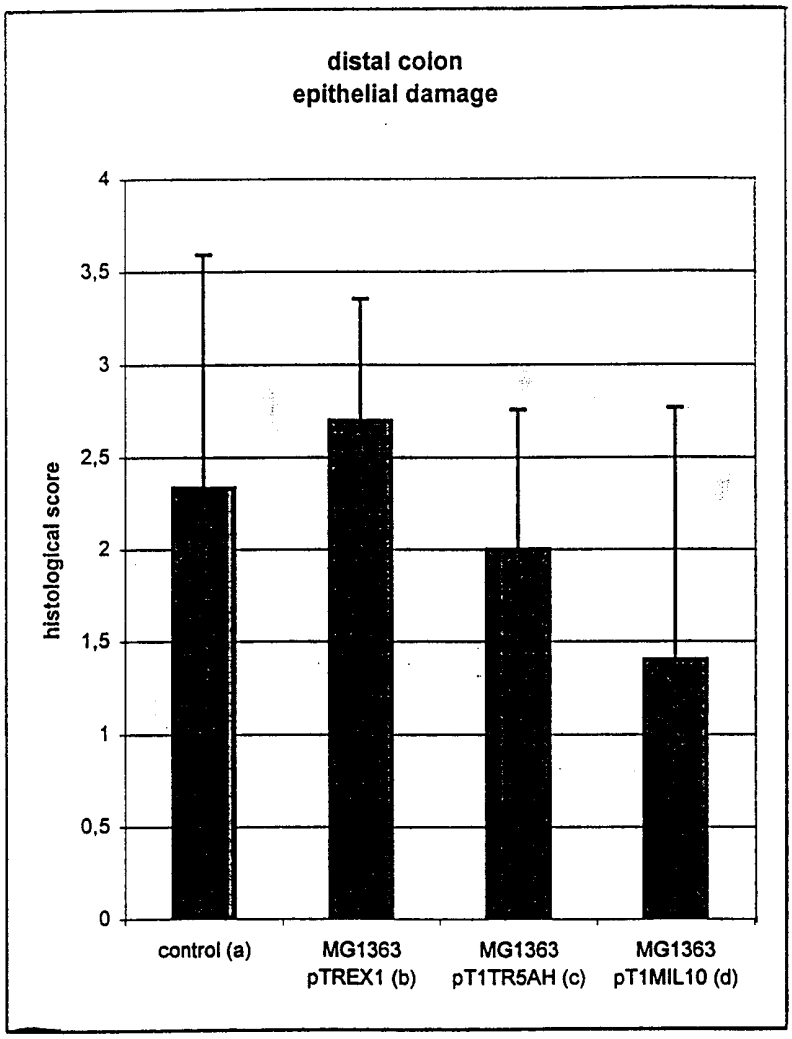

distal colon
epithelial damage
histological score
4
3,5
3
2,5
2
1,5
1
0,5
0
control (a)
MG1363 pTREX1 (b)
MG1363 pT1TR5AH (c)
MG1363 pT1MIL10 (d)

Figure 4

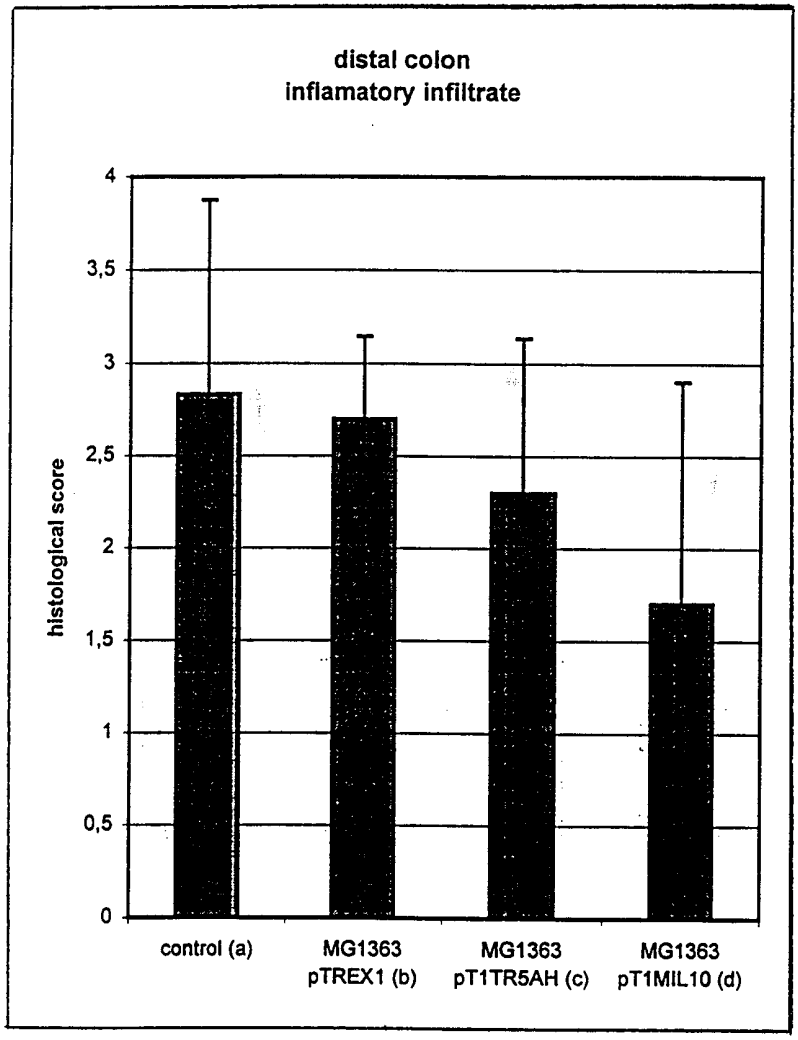

distal colon
inflamatory infiltrate
histological score
4
3,5
3
2,5
2
1,5
1
0,5
0
control (a)
MG1363 pTREX1 (b)
MG1363 pT1TR5AH (c)
MG1363 pT1MIL10 (d)

Figure 5

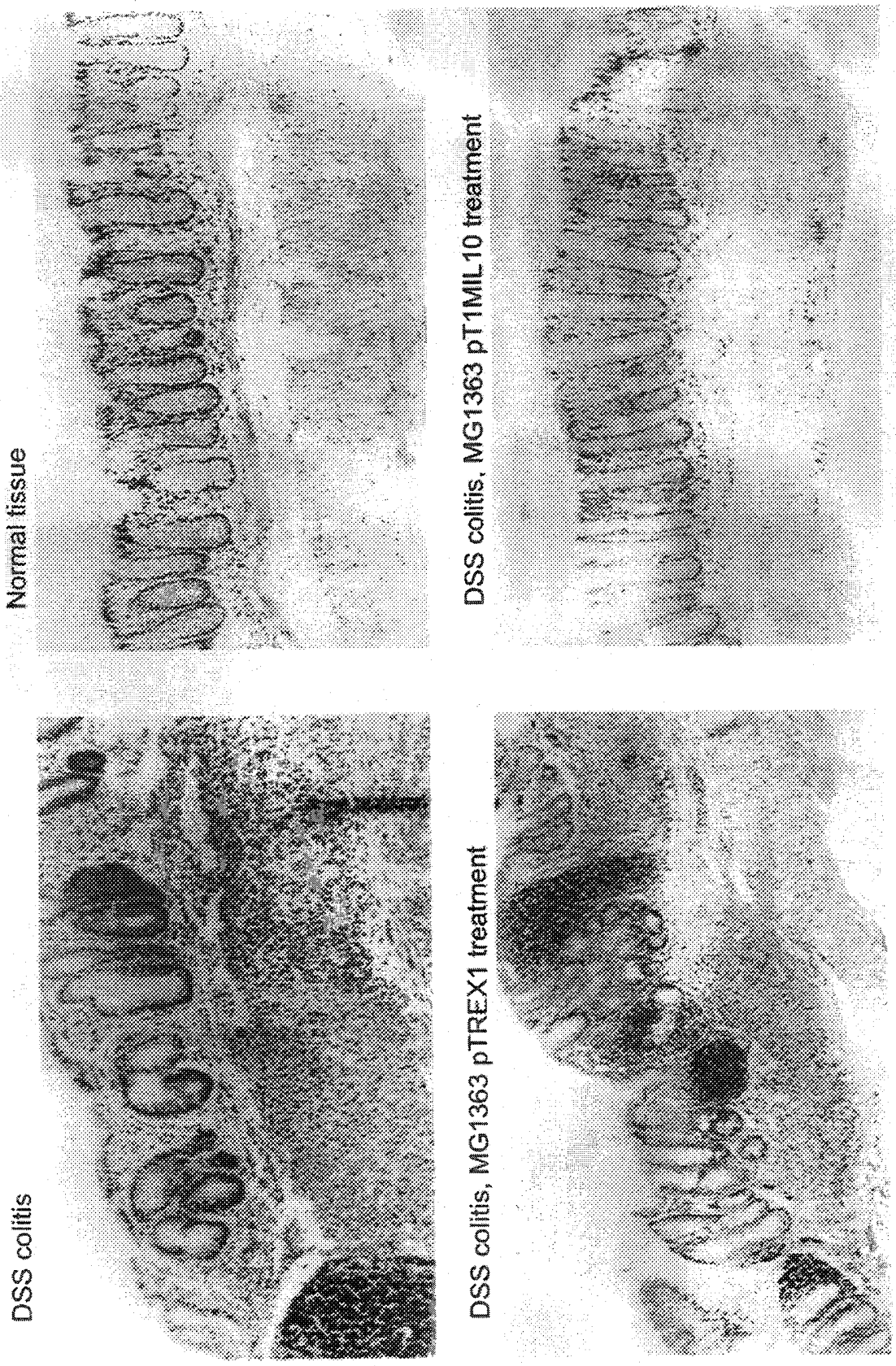

Normal tissue
DSS colitis, MG1363 pT1MIL10 treatment
DSS colitis
DSS colitis, MG1363 pTREX1 treatment

Figure 6

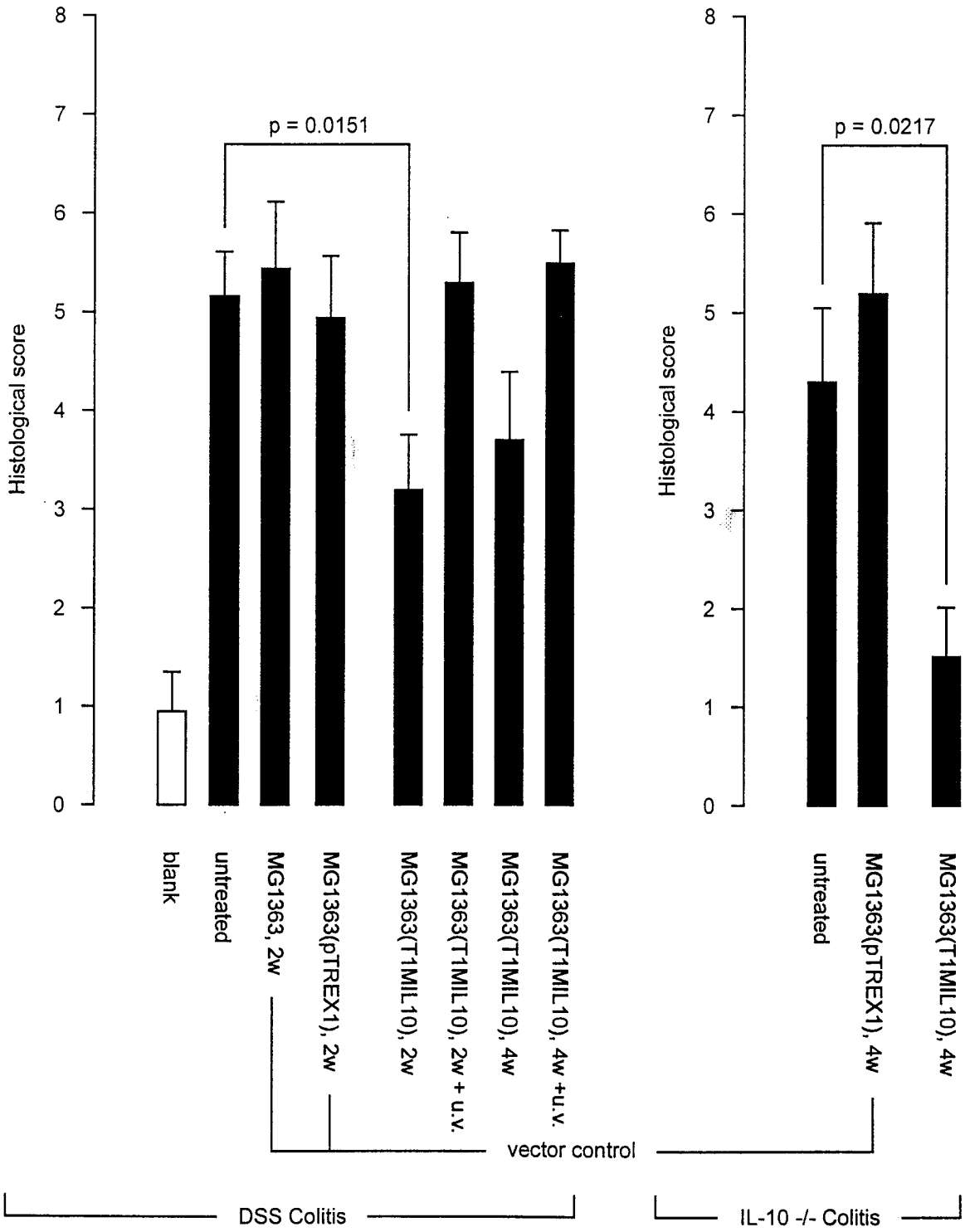
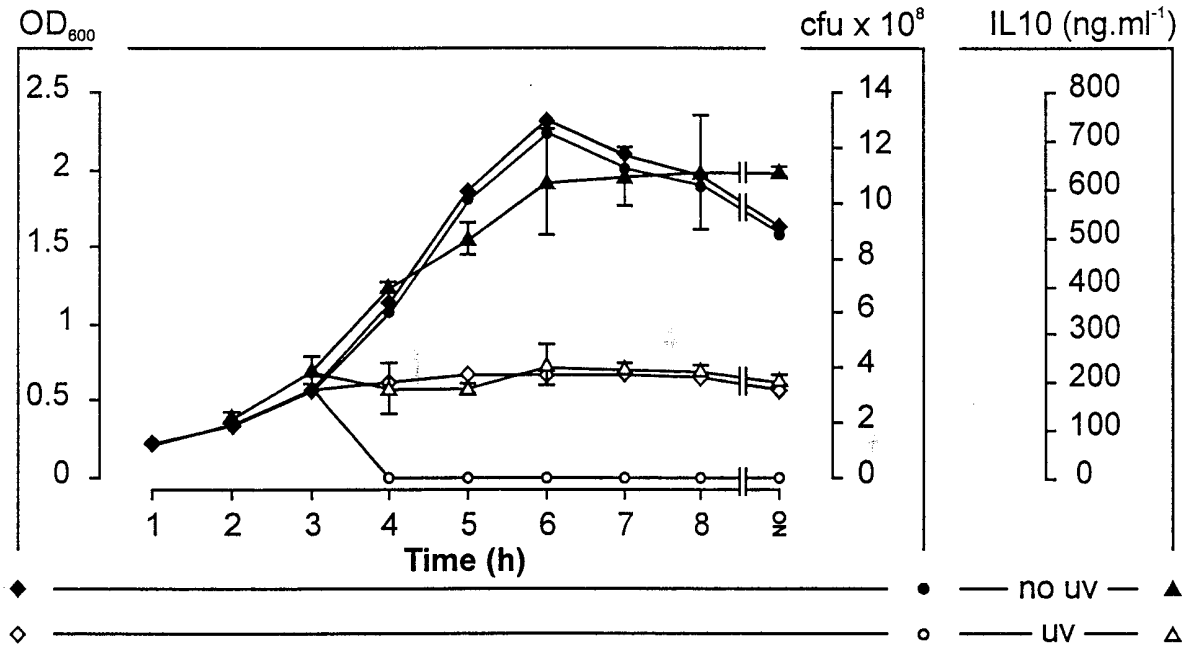

Histological score
0
1
2
3
4
5
6
7
8
p = 0.0151
p = 0.0217
blank
untreated
MG1363, 2w
MG1363(pTREX1), 2w
MG1363(T1MIL10), 2w
MG1363(T1MIL10), 2w + u.v.
MG1363(T1MIL10), 4w
MG1363(T1MIL10), 4w +u.v.
untreated
MG1363(pTREX1), 4w
MG1363(T1MIL10), 4w
vector control
DSS Colitis
IL-10 -/- Colitis

Figure 7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uv

Figure 8

SEQUENCE LISTING

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<120> USE OF CYTOKINE-PRODUCING LACTOCOCCUS STRAIN TO TREAT COLITIS

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