Mechanisms of Bacterial-Induced Experimental Colitis

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Frank Hoentjen

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IL-10 deficient bone marrow-derived dendritic cell stained for NF-κB (cRel) outside the nucleus under unstimulated conditions

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Mechanisms of Bacterial-Induced Experimental Colitis

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promotoren: prof.dr. C.J.J. Mulder
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Contents

Abbreviations

-/-	Deficient
ARE	Au-rich elements
BBE	Bacteroides bile esculin
BMDC	Bone marrow-derived dendritic cells
B. vulgatus	*Bacteroides vulgatus*
CARD	Caspase activation and recruitment domain
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFU	Colony forming units
COX-2	Cyclooxygenase-2
ChIP	Chromatin immunoprecipitation
CRP	C-reactive protein
DAPI	Diamidino-2-phenylindole
DSS	Dextran sulfate sodium
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
ERK	Extracellular signal-regulated protein kinase
FADD	Fas-activated death domain
FISH	Fluorescent *in-situ* hybridization
GR	Glucocorticoid receptor
HPA	Hypothalamic/pituitary/adrenal
HSP 60	Heat shock protein 60
IBD	Inflammatory bowel diseases
ICE	IL-1β converting enzyme
ICSBP	IFN consensus sequence-binding protein
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
IκB	Inhibitor of κB
IL-1RA	IL-1 receptor antagonist
IL-18BP	IL-18 binding protein
iNOS	Inducible nitric oxide synthetase
IP3	Inositol triphosphate
JAK	Janus Kinase
JNK	c-jun NH2 terminal kinase
KLH	Keyhole limpet hemocyanin
Lactobacillus GG	Lactobacillus rhamnosus GG
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinases
MPO	Myeloperoxidase
NK cells	Natural killer cells
NF-κB	Nuclear factor κB
PCR-DGGE	PCR-denaturing gradient gel electrophoresis
PG-PS	Peptidoglycan-polysaccharide
PKC	Protein kinase C
RAG	recombinant-activating genes

rnu/rnu	Homozygous nude (athymic)
rnu/+	Heterozygous nude (non-athymic)
SCFA	Short-chain fatty acids
SCID	Severe-combined immunodeficient
SOCS	Suppressor of cytokine signaling
Spp.	Species
STAT	Signal transducer and activator of transcription
TACE	TNFα cleavage enzyme
TCR	T cell receptor
TGF-β	Transforming growth factor β
T_H	T helper
TIR	Toll-IL-1-1R
TLR	Toll-like receptor
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
T_R	Regulatory T cells
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adapter protein

1 General Introduction

Clinical aspects of human inflammatory bowel diseases

Introduction

Crohn's disease and ulcerative colitis, collectively referred to as inflammatory bowel diseases (IBD), are chronic idiopathic inflammatory diseases of the gastrointestinal tract. IBD is generally regarded as a "Western" world disease and its frequency has increased considerably over the past few decades. Quality of life is severely affected in IBD patients, mainly due to the chronic relapses of disease. Complications such as stenoses and strictures are frequent in Crohn's disease, leading to multiple resections of affected parts of the gastrointestinal tract. In addition, chronic ulcerative colitis is associated with an increased frequency of colonic adenocarcinoma. Each of these factors highlights the importance of developing a better understanding of IBD.

The incidence of IBD has either continued to increase or has stabilized at a high rate in most developed countries. Even in less developed regions where IBD has been less common, the incidence is now increasing. This increase is the result of a combination of previously rising incidence and improved survival.[1] In 1998, a prospective study reported an incidence as high as 6.9 and 10.0 cases per 100,000 inhabitants per year of Crohn's disease and ulcerative colitis in Zuid-Limburg, The Netherlands, respectively.[2,3] The onset of both diseases occurs most frequently between the age of 15 and 30.

Crohn's disease

Although Crohn's disease and ulcerative colitis are both inflammatory disorders of the intestinal tract, they each have distinct patterns of symptoms and therapeutic strategies. Crohn's disease was first described in 1932 by Crohn, Ginsberg, and Oppenheimer as "ileitis regionalis", to be distinguished from intestinal tuberculosis.[4] Although Crohn's disease can occur at any location in the intestinal tract, the highest incidences are reported in the distal ileum, cecum, and right-sided colon. Clinical symptoms are diverse and involve nonbloody diarrhea, abdominal cramps, fever, weight loss, and perianal manifestations. Associated complications include fistula to skin and internal organs, strictures, and perirectal abscess formation. Gross appearance shows a thickened intestinal wall with a narrowed lumen, which can lead to bowel obstruction. In more advanced stages of the disease, the mucosa has a nodular appearance, often referred to as "cobblestones". Characteristic histopathologic features of Crohn's disease that do not occur in ulcerative colitis are transmural inflammation affecting all layers of the intestinal wall and mesenteric lymph nodes, and chronic non-caseating granulomatous inflammation. The intestinal tract shows a discontinuous pattern: severely affected regions alternate with normal parts, the so-called "skip-lesions". Current treatments for mild-moderate Crohn's disease include steroids, 5-aminosalicylic acid, and antibiotics. More severe and recurrent Crohn's disease requires azathioprine/6-mercaptopurine, methotrexate, and/or anti-TNF therapy. Surgical interventions are necessary to treat complications and drug-resistant patients.

Ulcerative colitis

Ulcerative colitis was first described by Wilks in 1859.[5] Ulcerative colitis is always restricted to the colon and involves the rectum. Major symptoms reflect colonic inflammation: diarrhea, rectal bleeding, and abdominal pain, often accompanied by fever and weight loss. The inflammation primarily involves the colonic mucosa, is uniform and continuous, and always progresses proximally. Pseudopolyps are commonly found during endoscopy. Initial microscopic

findings include goblet cell depletion, crypt hyperplasia, and neutrophilic infiltration. Chronic ulcerative colitis can lead to dysplasia, with increased risk for colorectal cancer in later stages of disease. Laboratory findings show perinuclear staining for antineutrophil cytoplasmic antibodies in 70% of ulcerative colitis patients. Medical treatment of ulcerative colitis includes systemic and topical steroids and 5-aminosalicylic acid for mild to moderate ulcerative colitis. More severe disease requires azathioprine/6-mercaptopurine or intravenous cyclosporine. Since ulcerative colitis is restricted to the colon, surgical treatment by total colectomy will cure the disease.

In this thesis, characteristics of experimental colitis are the main focus. Although the following sections will initially refer to mainly human IBD, during the course of this introduction background on experimental colitis will be provided as well.

Etiology of inflammatory bowel disease

Genetic factors

There is overwhelming evidence that genetic factors play an important role in IBD, and that the association is stronger for Crohn's disease than for ulcerative colitis. For example, 15-20% of all IBD patients have at least one affected relative, usually a first-degree relative. The absolute risk of IBD for first-degree family members is approximately 7%.[8,9] Disease concordance for IBD is higher in monozygotic twins (44-50%) than in dizygotic twins (0-4%).[8] These percentages are much lower for ulcerative colitis, indicating that genetic influences might be stronger in Crohn's disease than in ulcerative colitis. It is interesting that the concordance in monozygotic twins does not exceed 50%, indicating that environmental factors also play an important role in the onset of IBD. There are also differences in prevalence among the different ethnic groups. IBD is most frequent in Ashkenazi Jews, and high in Caucasians, but lower for Blacks. For example, mutations in the NOD2/CARD15 gene have been demonstrated to predict an earlier age-of-onset in Ashkenazi Jewish patients.[10] Progress in the field of genetics and in IBD research will continue to provide more insight into the variations observed between the different ethnic groups.

The absence of simple Mendelian inheritance patterns for predicting the risk of IBD development suggest that multiple genetic mutations are associated with IBD. The most important mutation discovered involves the gene on chromosome 16, encoding for NOD2/CARD15.[11,12] NOD2/CARD15 is an intracellular pattern-recognition receptor that recognizes muramyl dipeptide, a component of peptidoglycan-polysaccharide (PG-PS) present on bacterial cell walls.[13,14] NOD2 is present in numerous immune cells, including dendritic cells, monocytes, and epithelial cells.[15] It activates the nuclear factor κB (NF-κB) pathway, which is an intracellular signaling cascade crucial to the initiation of proinflammatory responses and protection against pathogens, as outlined in Chapter 2. Malfunction of NOD2 and the subsequent lack of NF-κB activation can lead to insufficient removal of pathogens.[16] Recently, this concept was revised using Card15$^{-/-}$ mice.[119] In the latter study, intact NOD2 signaling inhibited TLR-2-driven activation of NF-κB. Moreover, NOD2 deficiency or the presence of a Crohn's disease-like Card15 mutation increased TLR-2-mediated activation of NF-κB, and T_H1 responses were enhanced. Thus, CARD15 mutations may lead to disease by causing excessive T_H1 responses. This is one potential explanation for the relationship between NOD2/CARD15 mutations and the induction of inflammation.

Patients homozygote for the NOD2 mutation have a relative risk of 38 for developing Crohn's disease. This same factor is only 3 for heterozygotes.[12] However, some individuals who

are homozygote for the NOD2 mutation do not develop IBD and NOD2 polymorphisms only occur in a subgroup of patients.[17] Therefore, it is likely that susceptibility loci for IBD on other chromosomes are involved.[18] Of interest, two variants in the organic cation transporter cluster at 5q31, a previously reported locus (IBD5) associated with Crohn's disease, form a haplotype associated with susceptibility to Crohn disease.[120] These variants alter transcription and transporter functions of the organic cation transporters and interact with variants in CARD15, to increase the risk of Crohn's disease.

Increasing evidence suggests that genetic mutations may correlate with the phenotype of disease. This has been shown for location and aggressiveness of disease, and also for complications of IBD.[19] For example, common NOD2/CARD15 variants are associated with ileal disease and a fibrogenic, stenotic phenotype.[20-22] Furthermore, genes are involved in the efficacy and toxicity of therapy.[19] For example, expression of the glucocorticoid receptor β (GRβ) alters the corticosteroid response in IBD.[23] This receptor does not signal after binding to corticosteroids, in contrast to GRα which continues to signal. 83% of glucocorticoid-resistant ulcerative colitis patients expressed GRβ, whereas only 9% expressed GRβ in normal responders.

Genetic factors in the pathogenesis of IBD are supported by numerous animal models that have been developed using genetic manipulation. The genetically susceptible host develops intestinal inflammation due to the absence (e.g. IL-10 deficient (IL-$10^{-/-}$) mice) or overexpression (e.g. HLA-B27 transgenic rat) of specific genes.[24] Their wild-type littermates remain disease-free, emphasizing that a single genetic mutation can play a major role in the onset of severe inflammation. Animal models will be discussed in depth in the following paragraphs. In conclusion, identifying and understanding genetic mutations is crucial for predicting the onset of disease, resistance to therapy, and the risk factors for family members of patients with IBD.

Environmental factors

Evidence for environmental factors affecting the incidence of IBD is drawn from the increased incidence of IBD in the developed countries.[1,25,26] Dietary factors and Western public health greatly influence the incidence of Crohn's disease and ulcerative colitis. Additionally, the incidence of IBD changes when populations move between different regions. This is illustrated by the increasing incidence of IBD among Japanese immigrants in the United States.[27] There is also a decreased incidence of Crohn's disease among Ashkenazi Jews who moved from east-Europe to Israel.[28] More evidence for the influence of environmental factors is provided by the low concordance rates in monozygotic versus dizygotic twins, as discussed before.[8]

Environmental factors that can increase the risk of IBD include smoking, the use of NSAIDs, stress, and acute infections.[27] The best example of an environmental factor that influences IBD incidence is cigarette smoking. The risk of ulcerative colitis is increased for non-smokers, and especially for smokers who have recently quit,[29,30] whereas smoking increases the risk of Crohn's disease.[31] When combined with oral contraceptives, almost all IBD patients who smoke will experience exacerbation of disease. This figure is only 40% in patients who have only one of the two risk factors.[32] Therefore, although genetic susceptibility is required to develop IBD, environmental factors determine the onset and progression of disease.

Microbiological factors

Bacteria are crucial environmental factors that influence IBD. Many animal models of chronic intestinal inflammation have demonstrated the importance of the commensal intestinal bacteria for the onset and progression of disease. This is shown in HLA-B27/β2 microglobulin transgenic rats,[33,34] IL-$2^{-/-}$,[35] IL-$10^{-/-}$,[81] TCR$\alpha^{-/-}$,[36] SAMP-1/Yit,[37] and CD3ε_{26} transgenic mice[38] in

which the germ-free state prevents disease development and immune activation. The importance of intestinal bacteria in the pathogenesis of experimental colitis was further emphasized after showing that broad spectrum antibiotics can both prevent and treat colitis in HLA-B27 transgenic rats. Antibiotics have a similar effect in dextran sulfate sodium (DSS)-induced colitis in BALB/c mice[39] and in IL-10$^{-/-}$ mice.[40] Moreover, cecal bacterial overgrowth within an experimental blind loop exacerbates colitis in HLA-B27 transgenic rats, whereas a bypass of the cecum attenuates the disease in this model.[41] Thus, animal models of experimental colitis have shown that commensal bacteria and their products are required for induction and perpetuation of colitis.

Monoassociation studies have provided inside into the role of specific bacterial strains in the pathogenesis of colitis. Bacteria can be disease-inducing, neutral, or protective. For example, *Bacteroides vulgatus* (*B. vulgatus)* preferentially induces colitis in HLA-B27 transgenic rats after monoassociation for 4 weeks, whereas monoassociation with *Escherichia coli* (*E. coli*) does not cause disease.[42] Protective bacteria are called "probiotics" and one example of its protective effects is the prevention of inflammation by *Lactobacillus rhamnosus GG* (*Lactobacillus GG)* in HLA-B27 transgenic rats.[43] Host-specificity has been shown by the observation that *E. coli* induces colitis in IL-10$^{-/-}$ mice but not in HLA-B27 transgenic rats.[42,44] Moreover, regional specificity is evident for different bacteria. *Enterococcus faecalis* (*E. faecalis)* induced mild distal colitis, whereas *E. coli* caused a cecal inflammation in IL-10$^{-/-}$ mice.[44]

Many studies have attempted to locate pathogens associated with human IBD. One bacterial species associated with IBD is *E. coli*. Its role in chronic intestinal inflammation was demonstrated by the observation that ileal mucosa from Crohn's patients with postoperative recurrence contained more enteroadherent/invasive *E. coli* strains than healthy controls.[45] This association only applied to the ileum of healthy and diseased Crohn's disease patients, but not for colonic Crohn's disease nor ulcerative colitis patients.[46] In addition, IBD patients have increased serum and mucosal antibody responses to several commensal bacteria, including *E. coli.*[47-49] T-cell clones from mucosal biopsies showed specific responses to selective commensal bacteria, including *Bacteroides*, *Bifidobacteria*, and *E. coli.*[50,51]

Studies in animal models and human IBD patients highlight the importance of commensal bacteria for the induction and perpetuation of IBD. The balance between protective and disease-inducing bacteria determines the effects on host homeostasis. While significant steps have been made to identify those bacteria, more than half of the bacteria found in the colon are not cultivable and therefore difficult to study. Further studies are needed to investigate the role of individual commensal bacterial strains in the pathogenesis of IBD.

Immunological factors

The presence of pathogenic and non-pathogenic microorganisms induces innate and adaptive immune responses and lead to the activation of a complex gene program aimed at re-establishing host homeostasis. The initiation of innate immunity is a critical feature of host homeostasis, and failure to regulate and/or terminate this response can have deleterious consequences for the host. For example, IBD is associated with both dysregulated innate and adaptive immune responses to luminal non-pathogenic bacteria.[24,52-54] The following sections will introduce both innate and adaptive immunity in IBD. Additionally, in Chapter 2, a more extensive overview of innate immune responses is provided.

Innate immune system

From an evolutionary perspective, the innate immune system is an old system, well conserved in plants and animals. It functions as a first line of defense against pathogens, and uses

toll-like receptors (TLR) as mediators of innate immune responses.[55] Bacterial products act as ligands for TLR, and subsequently activate various transcription factors, such as NF-κB. This activation results in cytokine secretion and upregulation of costimulatory molecules that alter adaptive immune responses, leading to regulatory or proinflammatory immune responses. This link between the innate immune system and the adaptive immune system is of high relevance to IBD, because in IBD patients it is well known that commensal bacteria activate the innate immune system. In turn, Crohn's disease is a T helper-1 (T_H1)-mediated disease, driven by T lymphocytes that are partially induced by innate immune responses. Important cells and their cytokines involved in innate immune responses, such as epithelial cells, dendritic cells, and macrophages, are discussed in Chapter 2.

Adaptive immune system

Immune cells from the lymphoid lineage, which include B and T lymphocytes, respond to stimuli in an antigen-specific manner. This feature allows the adaptive immune system to respond in a highly specific manner to a tremendous variety of antigens present in the intestinal lumen. Regulating these responses is crucial to ensure the intestinal homeostasis. This is highly relevant to IBD, since the commensal microflora drives immune responses and therefore requires tight regulation. Dysregulation can have deleterious consequences, as shown in various animal models, such as the IL-$10^{-/-}$ mouse model.

T cells recognize a wide variety of antigens presented by antigen-presenting cells using the T cell receptor (TCR) repertoire. The TCR forms a complex with antigens, followed by activation of a cascade resulting in the secretion of proinflammatory cytokines by effector T cells, categorized by T_H1 and T_H2 responses. T_H1 responses are characterized by increased secretion of interferon-γ (IFN-γ) and tumor necrosis factor (TNF), whereas T_H2 responses involve secretion of IL-4, IL-5, and/or IL-13. Uncontrolled secretion of these cytokines by T cells can induce inflammation. Interleukin (IL)-12, IL-18 and IL-23 from antigen-presenting cells stimulate T_H1 lymphocytes, while IL-10 activates regulatory cells. In genetically engineered animal models, T cells have been shown to be crucial to the development of inflammation. This has been demonstrated in IL-$2^{-/-}$ mice,[56] IL-$10^{-/-}$ mice,[57] TCR$\alpha^{-/-}$ mice,[58] Tgε_{26} mice,[59] and transfer of either CD45RB^{high} or T cells into either severe-combined immunodeficient (SCID) mice[60] or athymic rats,[61] respectively. In the HLA-B27 transgenic rat model, Breban et al previously showed that nude athymic (*rnu/rnu*) HLA-B27 transgenic rats lacking T cells do not develop colitis under specific pathogen-free conditions, and that $CD4^+$ T cells can transfer disease.[62]

In human IBD, T_H1 responses are mainly detected in Crohn's disease patients, whereas ulcerative colitis is characterized by activation of T_H2-like or IL-13 NK T cell mediated pathways.[121] Therapeutic approaches targeting T cells have shown their efficacy. For example, anti-TNF therapy can attenuate intestinal inflammation by induction of apoptosis in lamina propria T-lymphocytes of patients with Crohn's disease.[63]

However, T cells also have regulatory functions. Regulatory T (T_R) cells secrete IL-10 and transforming growth factor β (TGF-β) that are capable of regulating inflammatory responses. The importance of these cytokines has been demonstrated by the fact that TGF-$\beta^{-/-}$ mice die within five weeks of severe multiple organ inflammation,[64] and the severe intestinal inflammation that develops in IL-$10^{-/-}$ mice.[65]

Several subsets of T cells contribute to regulation of immune responses. $CD4^+$ T_R cells constitutively express CD25 on their surface and induce T_H3 responses by secreting TGF-β. Studies in animal models have helped to identify the regulatory functions of $CD4^+$ T cells. In an elegant study, Powrie et al showed that transfer of $CD4^+$CD45RB^{hi} T cells from normal mice

induced colitis in SCID mice. This inflammatory process could be prevented by co-transfer of $CD4^+$ cells that express low levels of CD45RB from normal mice.[60] The same principle was shown in the athymic rat, where the transfer of T cells that express high levels of the CD45 isoform, designated CD45RC, mediated inflammation in several different organ systems, and the severity of inflammation was greatly reduced by co-transfer of a $CD4^+CD45RC^{lo}$ cell population.[61] The mechanisms of T_R cell development has been unknown for a long time. However, recently Foxp3 has been recognized as a key regulatory gene for the development of these regulatory T cells.[66] This finding allows better distinction of T_R cells and provides more insight in the development of a naïve T cell into a T_R cells.

A second example of a subset of regulatory T cells are T_R1 cells that secrete IL-10. T_R1 cells inhibited colitis in SCID mice after transfer of $CD4^+CD45RB^{high}$ cells, mediated by the secretion of IL-10.[67] Thus, different subsets of T cells can induce either inflammatory or regulatory responses to intestinal bacteria. The ratio of these two responses will determine the outcome: homeostasis or inflammation.

Until recently, the role of B cells in the mechanisms of colitis was thought to be minor. However, in recent years, reports showed that B cells are not merely bystanders, but actively participate in onset and perpetuation of disease. B cells have numerous immune functions, such as production of immunoglobulins (Igs) and cytokines, antigen presentation, and the regulation of dendritic cell function.[68-70] Similar to the distinction between proinflammatory and regulatory T cells, B cells also have distinct functions.[71] B cells are required for the development of several autoimmune disorders by the secretion of pathogenic Igs.[71] In contrast, Mizoguchi et al demonstrated that IL-10-producing B cells expressing CD1d are protective, since B cell/TCRα double deficient mice had more colitis than TCRα deficient mice with competent B cells.[72,73] The role of B cell-secreted IL-10 was further demonstrated by the observation that the transfer of B cells from IL-10/TCRα double deficient mice was unable to suppress chronic intestinal inflammation in B cell/TCRα double deficient mice.[72] Thus, T cells are required for the induction of colitis in many models of experimental inflammation, but T and B cells can also regulate inflammation. Although not fully understood, the fine interplay between the several subsets of antigen-presenting cells, and B and T cells, determines the outcome of bacterial-induced responses.

Animal models

Introduction

Animal models of experimental colitis have contributed to the enormous progress made in the last decade to our further understanding of IBD. Although there are no animal models that identically replicate human IBD, numerous characteristics can be found in each individual model, and the combination of knowledge acquired helps us to better understand IBD. One way of categorizing animal models of intestinal inflammation is to distinguish between induced and spontaneous colitis. Chemical-induced colitis is reproducible and relatively easy to perform, however, only certain characteristics are relevant to human IBD. Chemical inducers of colitis include DSS, acetic acid, and trinitrobenzenesulfonic acid (TNBS).[74]

Genetically engineered models resemble human IBD better than chemically induced models of colitis. These models illustrate the relevance of specific genes for the development of chronic intestinal inflammation. In order to accomplish this, genes of interest are either deleted,

'knock-out', or inserted, 'knock-in'.[24] Two models of spontaneous colitis are extensively used throughout this thesis: IL-10$^{-/-}$ mice and HLA-B27 transgenic rats.

HLA-B27 transgenic rats

HLA-B27 transgenic rats overexpress the human MHC class I molecule HLA-B27 and β2-microglobulin. Under specific pathogen-free conditions, these animals develop spontaneous colitis and gastritis starting at 10 weeks of age, while their non-transgenic littermates remain disease-free.[75] Disease-susceptible transgenic animals have diarrhea, and often display signs of arthritis and uveitis. Inflammation is characterized by crypt hyperplasia, mucosal thickening, mononuclear cell infiltration, and crypt abscesses in later stages of disease.

Studies in *rnu/rnu* rats, which are congenitally athymic and do not have T lymphocytes, have helped us better understand the development of T_H1 mediated colitis in this model. Even if they carry the disease-prone B27 transgenic locus *rnu/rnu* transgenic rats are protected from disease, whereas the presence of T cells in their heterozygous nude (*rnu/+*) transgenic littermates coincides with development of colitis under specific pathogen-free conditions.[62] The critical role of T cells in inducing intestinal inflammation in this model was further emphasized by the induction of colitis by the transfer of T cells into specific pathogen-free *rnu/rnu* HLA-B27 transgenic recipients, with CD4$^+$ T cells being more efficient than CD8$^+$ T cells in transferring disease.[62]

HLA-B27 rats raised in a germ-free environment fail to develop intestinal inflammation and arthritis,[33,34] however, they will develop colitis and gastritis within one month after transfer to a specific pathogen-free environment and colonization with nonpathogenic commensal bacteria.[34] More evidence for the role of commensal bacteria in the pathogenesis of colitis in this model is provided by the prevention and treatment of colitis by broad spectrum antibiotics.[39] Furthermore, monoassociation studies showed selectivity for the induction of colitis. *B. vulgatus* induced colitis, whereas *E. coli* did not induce disease.[42] *Lactobacillus GG* prevented recurrence of disease after antibiotic treatment, indicating that bacteria can also have beneficial effects in this model.[43]

The HLA-B27 rat model represents a larger group of diseases, referred to as spondyloarthropathies.[76] This group includes colitis, arthritis, orchitis, and psoriasis-like skin lesions.[75,77] In patients, the overexpression of HLA-B27 does not lead to colitis, but it does increase the risk of extra-intestinal manifestations. The overexpression of a gene involved in human disease, and extra-intestinal manifestations of disease, make this rat model therefore highly relevant for human IBD.

IL-10$^{-/-}$ mice

Deletion of the gene encoding IL-10 causes the absence of production of the immunoregulatory cytokine IL-10, and leads to T_H1-mediated colitis. IL-10$^{-/-}$ mice develop predominantly cecal inflammation shortly after weaning when susceptible strains (129 SvEv or mixed 129/C57Bl/6 background) are colonized with specific pathogen-free bacteria.[65,78] As disease progresses, inflammation develops in the ascending and transverse colon, and finally in the remainder of the colon and rectum. Rectal prolapses and diarrhea are frequently observed. Histologic features of chronic intestinal inflammation include crypt hyperplasia, neutrophilic infiltrations, ulcers, and crypt abscesses.

T cells are required for the induction of intestinal inflammation in this model as the transfer of CD4$^+$ T cells from IL-10$^{-/-}$ donors into recombinant-activating genes deficient (RAG$^{-/-}$) mice induced colitis.[57] B cells are not required, as similar colitis developed in B cell$^{-/-}$IL-10$^{-/-}$ mice versus IL-10$^{-/-}$ mice.[57] The proinflammatory cytokine IL-12 is a key mediator of inflammation,

since IL-12 blocking antibodies prevented disease in IL-10$^{-/-}$ mice.[79] Interestingly, recombinant IL-10 prevents the onset of disease, but does not treat established colitis.[57] The molecular mechanisms for the immunoregulatory effects of IL-10 are largely unknown. Therefore, the IL-10 deficient mouse provides a model of experimental colitis in which the role of endogenous IL-10 can be studied.

Bacteria and their products are required for inflammation in IL-10$^{-/-}$ mice. Conventially housed IL-10$^{-/-}$ mice develop lethal enterocolitis, whereas specific pathogen-free conditions result in aggressive, non-lethal inflammation.[65] Monoassociation studies demonstrated that single bacterial strains induce phenotypically different colitis. *E. faecalis* induced a mild distal colitis with a slow onset of disease while *E. coli* induced rapid intestinal inflammation, predominantly in the cecum.[44,80] Germ-free IL-10$^{-/-}$ mice do not develop disease, and do not show any signs of immune activation.[81]

Also antibiotics can prevent disease,[40] a finding used in Chapter 3 to study to local effects of antibiotics in more detail. Bacteria also exert protective effects, as demonstrated by attenuation of disease by probiotics in murine models of colitis.[82,83] Thus, bacteria are required in IL-10$^{-/-}$ mice to develop a T_H1-mediated colitis with increased IL-12 secretion and activated CD4$^+$ T cells.

Manipulating the intestinal microflora

Introduction

Under normal conditions intestinal homeostasis is well maintained in the presence of commensal non-pathogenic bacteria. However, in the genetically susceptible host, chronic intestinal inflammation can develop in response to the same residential intestinal bacteria.[24] Luminal bacteria are present in high concentrations, as high as 10^{11} organisms/gram luminal contents.[84] Moreover, Crohn's disease occurs at sites with the highest levels of anaerobic bacteria. Manipulating the intestinal microflora may be of importance in changing the natural course of colitis. Treatments that target the bacterial load and the composition of the luminal microflora include antibiotics, probiotics, and prebiotics.

Antibiotics

Broad spectrum antibiotics have proved to be beneficial in treating chronic intestinal inflammation. Most clinicians use broad spectrum antibiotics for septic complications, and as adjuvant therapy in fulminant colitis and toxic megacolon. Selective antibiotics are used mainly in Crohn's disease. In Crohn's disease, metronidazole was effective in treating colonic inflammation.[85,86] Metronidazole in combination with ciprofloxacin is preferentially used for colonic inflammation.[87-89] Although controversial, ciprofloxacin has been used in clinical trials and treated Crohn's disease.[90,91] In both fistulizing Crohn's disease and in pouchitis there is a role for metronidazole.[92,93,94,95] Only a few well-designed clinical trials support the use of antibiotics in ulcerative colitis patients.[96,97]

Most studies in animal models of colitis support the use of antibiotics. A combination of metronidazole and neomycin prevented and treated colitis in IL-10$^{-/-}$ mice.[40] Vancomycin-imipenem prevented and treated colitis in HLA-B27 rats, DSS-induced colitis in mice [39], and TNBS-induced experimental colitis in rats.[98] The combination of ciprofloxacin and metronidazole prevented and treated inflammation in the SAMP1/Yit spontaneous ileitis model [99] and it improved acute but not chronic DSS-induced colitis in mice.[100] An interesting observation from studies in animal models is that antibiotics are more effective in prevention than treatment of

chronic colitis. Ciprofloxacin prevented the induction of colitis in IL-10$^{-/-}$ mice born under specific pathogen-free conditions, but showed only minor effects in established colitis.[40] Moreover, in HLA-B27 transgenic rats and DSS-treated mice, oral administration of either ciprofloxacin or metronidazole prevented colitis but was less effective in treating established inflammation.[39]

Probiotics

Probiotics are living commensal microorganisms that are important to the health and well-being of the host.[101] The most convincing evidence so far is derived from a clinical trial with VSL#3. VSL#3 is a cocktail of eight different probiotic species that was very effective in preventing chronic pouchitis after antibiotic-induced remission,[102] and in treating ulcerative colitis patients.[103,104] Single probiotic species have also been identified and shown to improve disease in ulcerative colitis patients, as shown for the *E. coli* 1917 Nissle.[105]

Certain probiotic bacteria, such as *Lactobacillus* species, showed protection in several experimental models of chronic intestinal inflammation, including specific pathogen-free HLA-B27 transgenic rats and IL-10$^{-/-}$ mice.[43,82,83] Interestingly, the specificity of probiotics and the background of the host is important. *Lactobacillus GG* prevented colitis in HLA-B27 transgenic rats but not in IL-10$^{-/-}$ mice. In contrast, *Lactobacillus plantarum* was effective in IL-10$^{-/-}$ mice but had no effect in HLA-B27 transgenic rats after antibiotic treatment.[43,83]

Relatively little is known about the mechanisms for the protective effects of probiotic bacteria. The protective mechanisms suggested can be broadly categorized into three groups. First, probiotics exert their beneficial effects by suppression of growth or function of pathogenic bacteria. *Bifidobacteria infantis* protected the gut epithelial layer from being invaded by *Bacteroides*, thereby suppressing the systemic antibody response raised by *Bacteroides.*[106] Second, probiotics can improve the intestinal barrier function. This was demonstrated by the decreased epithelial permeability in IL-10$^{-/-}$ mice after treatment with VSL#3.[82] Third, probiotics have immunoregulatory activities. Dieleman et al showed increased cecal IL-10 and decreased IL-1β secretion after *Lactobacillus GG* treatment in HLA-B27 transgenic rats.[43] Also, non-viable probiotic DNA isolated from the probiotic cocktail VSL#3 attenuated intestinal inflammation in IL-10$^{-/-}$ mice, which effect was mediated by TLR-9.[107] The clinical use for probiotics is emerging, and the advances made in explaining the mechanisms of probiotics are promising.

Prebiotics

Prebiotics are non-digestible food ingredients which beneficially affect the host by selectively stimulating growth, activity, or both of selective intestinal (probiotic) bacteria.[108] Prebiotics are easy to administer and do not require live bacteria. Because of these characteristics prebiotics can be of value for treatment of IBD. However, clinical experience with prebiotic treatment is very limited. Only few small clinical trials have demonstrated the efficacy for prebiotics in human IBD. For example, germinated barley food extracts decreased clinical and endoscopic inflammation in mild-moderate ulcerative colitis.[109,110]

Animal models of inflammation have provided some insight in the effects of prebiotics. In rats, oral inulin decreased distal colonic lesions in DSS-induced colitis,[111] and oral fructo-oligosaccharides decreased TNBS-induced colitis, although its effects were apparent only after 14 days of feeding.[112] No effects were seen after oral administration of trans-galacto-oligosaccharide started before or during TNBS-colitis induction, indicating that prebiotics are not equally effective in preventing disease.[113]

Oral prebiotics administration is associated with increased luminal concentrations of probiotic bacteria, such as *Bifidobacteria* and *Lactobacilli* species.[109,111,112,114,115] Adding *Lactobacilli* and *Bifidobacteria* to topical butyrate solutions in rats with TNBS-induced colitis was necessary to induce a similar improvement as shown by oral fructo-oligosaccharides administration suggesting that increased concentrations of probiotic bacteria mediated the clinical efficacy of prebiotics.[112] A second potential mechanism for prebiotics to attenuate colitis is increased levels of short-chain fatty acids (SCFA). The end-products of prebiotic fermentation by commensal organisms present in the colon lead to the production of lactate and SCFA. This results in increased acidity, which may inhibit the growth of disease-inducing bacteria.[112] Changes in SCFA production, especially increased luminal butyrate concentrations can also affect proliferation and differentiation,[116] tight junction permeability[117] and epithelial restitution.[118] Moreover, butyrate exerts immunomodulatory effects and decreases NF-κB activity.[109] Taken together, although progress has been made in the last years, our understanding of prebiotics is still in its infancy.

Outline

As described in the introduction, the pathogenesis of inflammatory bowel disease involves an overly aggressive immune response to commensal luminal bacteria. Therefore, this thesis addresses

1. The attenuation of bacterial-induced experimental colitis by manipulating the intestinal microflora.
2. The regulation of bacterial-induced innate and adaptive immune responses.

Chapter 1 and 2 give an overview of genetic, microbiological, and immunological aspects of the pathogenesis of IBD. It is concluded that IBD is now considered an overly aggressive immune response of the genetically susceptible host to luminal commensal bacteria. Various treatment strategies are discussed that can alter the microflora, thereby preventing and treating experimental colitis. Furthermore, the importance of various cell types, cytokine secretion, and signaling pathways in innate and adaptive immunity is explained.

Part I describes attenuation of colitis by manipulating the intestinal microflora. In Chapter 3 the effect of selective and broad spectrum antibiotic treatment was studied in IL-$10^{-/-}$ mice. Aim was to assess the specific local effects in the intestinal tract exerted by the various antibiotic treatments. Fluorescent *in situ* hybridization (FISH) and bacterial cultures were used to quantify specific bacterial strains before and after treatment, and the severity of inflammation was determined by gut cultures and histology scores. In Chapter 4, we investigated if the probiotic *Lactobacillus GG* can prevent colitis in HLA-B27 transgenic rats monoassociated with *B. vulgatus*. Furthermore, we intended to determine if *Lactobacillus GG* or *Lactobacillus plantarum* can treat established colitis in specific pathogen-free transgenic rats and prevent recurrent disease after antibiotics were stopped. Prior to monoassociation with *B. vulgatus,* HLA-B27 transgenic rats were colonized with *Lactobacillus GG* or no bacteria. Secondly, specific pathogen-free HLA-B27 transgenic rats received oral vancomycin-imipenem for 2 weeks, or water alone, followed by 4 weeks treatment with either oral *Lactobacillus GG, Lactobacillus plantarum* or water only. Disease activity was quantified by blinded gross and histologic scores, cecal myeloperoxidase (MPO), and the proinflammatory cecal cytokines IL-1β and TNF, and the regulatory cytokines

TGF-β and IL-10. Chapter 5 describes the effects of prebiotic treatment on colitis in HLA-B27 transgenic rats. Aim was to investigate the course of colitis and to identify protective mechanisms of the prebiotic combination inulin/oligofructose. Cecal IL-1β and MPO, mesenteric lymph node cell cytokine secretion, and histology scores were used to assess inflammation. FISH and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) were used to quantify specific bacterial strains before and after treatment.

In Part II, bacterial-induced innate and adaptive immune responses in HLA-B27 transgenic rats and IL-$10^{-/-}$mice were studied. Chapter 6 describes cytokine profiles in mesenteric lymph node cells from HLA-B27 transgenic rats. The ratio of proinflammatory cytokines (IFN-γ, IL-12) versus anti-inflammatory cytokines (IL-10, TGF-β) can determine the outcome of immune responses. Therefore, aim of this study was to characterize the different subsets of cells and their cytokine secretion in response to commensal bacteria in transgenic and non-transgenic HLA-B27 rats. Chapter 7 was designed to investigate whether athymic *rnu/rnu* HLA-B27 transgenic rats develop colitis in a model of monoassociation with the non-pathogenic bacterial strain *B. vulgatus*. The requirement of T cells for induction of disease was tested by transfer of $CD4^+$ T cells into athymic recipients that do not have T cells. Inflammatory parameters were measured, such as cecal IL-1β and MPO, mesenteric lymph node cell cytokine secretion, and histologic scores. In Chapter 8 we used adoptive transfer of immune cells into athymic *rnu/rnu* HLA-B27 transgenic rats to investigate the role of the antigen presenting cells carrying the HLA-B27 molecule on the onset of disease. Non-transgenic and transgenic mesenteric lymph node cells were injected into nude non-transgenic and transgenic recipients lacking T cells. Development of colitis was assessed by cecal IL-1β and MPO, mesenteric lymph node cells cytokine secretion, and histologic scores. Labeling of donor cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) allowed us to monitor cell proliferation in recipients over time. In Chapter 9, bacterial-induced innate immune responses were studied. Bone marrow-derived dendritic cells (BMDC) from wild-type and IL-$10^{-/-}$ mice were used to investigate the molecular mechanisms of the dysregulated IL-12 responses in IL-$10^{-/-}$ mice compared to their wild-type littermates. Activation of the NF-κB pathway, and the role of IL-10-activated signal transducer and activator of transcription (STAT) 3 were investigated.

Chapter 10 contains the general discussion. After the introduction, the effects of manipulating the microflora in models of experimental colitis are discussed. Furthermore, adaptive immune responses and innate immune responses in experimental colitis are discussed.

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Proinflammatory cytokines and signaling pathways in intestinal innate immune cells

2

R. Balfour Sartor[1] and Frank Hoentjen[1,2]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

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Introduction

Activated innate immune cells mediate a variety of protective and proinflammatory mucosal immune responses with key roles in clearance of microbial pathogens, recruitment of effector cells into the inflammatory focus, tissue destruction and remodeling, and antigen presentation. These activities are relevant to both acute and chronic inflammatory processes at mucosal surfaces, including IBD, infection, asthma, periodontal disease, bronchitis and cystic fibrosis. Inflammatory cytokines, most notably IL-1, TNF, IL-6, and multiple chemokines, are secreted by a variety of activated innate immune cells of hematologic, mesenchymal, epithelial and endothelial origin (table 1). In contrast, the T_H1 polarizing cytokines IL-12, IL-18 and IL-23 are more selectively secreted by antigen presenting cells, such as dendritic cells, macrophages, and in the case of IL-18, epithelial cells.

Cell type	Function	Primarily cytokine profile
Bone marrow-derived		
Dendritic cells[1]	APC	IL-10, 12, 18, 23, T cell, chemokines
Neutrophil	Phagocytic, effector	IL-1, 6, TNF
Eosinophil	Effector, phagocytic	IL-1, TNF
Mast cell	Effector	IL-1, IL-6, TNF
Natural killer cell	Effector	IFN-γ
Epithelial origin		
Epithelial	Effector, barrier function, APC	Chemokines, IL-18, IL-1RA
Paneth cell	Effector	
Mesenchymal origin		
Smooth muscle, fibroblast, myofibroblast	Motility, matrix secretion, tissue remodeling, effector	IL-1, 6, TNF, chemokines
Endothelial cell	Adhesion molecules, effector	IL-1, 6, TNF, chemokines

Table 1. Secretion of proinflammatory cytokines by mucosal innate immune cells.
[1] dendritic cell subsets: myeloid, lymphoid, plasmacytoid

Many microbial products and proinflammatory molecules activate innate immune responses, culminating in increased transcription and secretion of proinflammatory cytokines. Microbial products stimulate cells through a family of pattern recognition receptors that induce signals through NF-κB and mitogen activated protein kinases (MAPK). In addition, innate immune cells can be activated by proinflammatory cytokines (IL-1 and TNF), reactive oxygen metabolites and products of the coagulation and complement cascades.

Maintenance of mucosal homeostasis depends on the ability of activated innate immune cells to appropriately downregulate the inflammatory response to microbial and inflammatory signals through secreted and cytoplasmic regulatory molecules (table 2). Mechanisms of inhibition include suppressing responses to inflammatory signals by downregulating membrane receptors, i.e. CD14 and TLR, which promotes unresponsiveness to ubiquitous stimuli by

emigrating monocytes and maturing epithelial cells, as well as upregulation of suppressive molecules, including PPARγ, prostaglandins, suppressor of cytokine signaling (SOCS) and A20.

Molecule	Function	Primarily Induced By
Secreted		
IL-10	Inhibits macrophage and APC activation, induces SOCS3	IL-1, TNF, IFN-γ, CD40L
Anti-IL-1 receptor	Inhibits IL-1α & β binding	IL-1, TNF
PGE_2, PGI_2, PGJ_2	Inhibits macrophage activation	IL-1, TNF
Corticosteroids	Blocks NF-κB, chemotaxis	IL-1, TNF, IL-6
IL-1RII	Binds IL-1 without signaling	IL-4
TNFRI (p55)	Binds TNF, LTα without signaling	IFN-α
Cytoplasmic		
A20	Inhibits TNF signaling	TNF
IκBα	Inhibits NF-κB activation	IL-1, TNF, bacterial products (NF-κB activation)
PPARγ	Inhibits NF-κB activation	Bacterial products, (NF-κB activation)
COX 2	Inhibits macrophage activation	IL-1, TNF, IL-6, bacterial products (NF-κB activation)
iNOS	Inhibits NF-κB activation	IL-1, TNF, IL-6, bacterial products (NF-κB activation)

Table 2. Regulatory molecules induced by proinflammatory cytokines.

This chapter describes the biologic activities, signaling pathways, and regulatory mechanisms of the most common proinflammatory cytokines. The reader is referred to other reviews on this topic.[1,2]

Profile of cytokines secreted by innate immune cells

Introduction

Bone marrow-derived, epithelial, mesenchymal and endothelial innate immune cells each secrete a unique characteristic profile of cytokines, inflammatory mediators and antimicrobial peptides when stimulated with a variety of microbial products (table 1). Due to conserved signaling pathways proinflammatory cytokines induce expression of identical molecules in different cells, thereby amplifying the inflammatory response in cells not exposed to the original inciting agent and also broadening the repertoire of activating signals. Moreover, members of

cytokine families are usually stimulated or suppressed in concert via redundant transcriptional regulatory pathways.

Epithelial cells

Mucosal epithelial cells act as sensors of microbial invasion and secreted toxins by liberating chemokines and, to a lesser degree, proinflammatory molecules.[3] These molecules activate protective mucosal inflammatory and immune responses by stimulating the emigration and activation of a variety of effector cells to respond to the inciting stimulus. Expression of chemotactic signals is temporally and spatially coordinated, leading to the emigration of first neutrophils, then monocytes and finally lymphocytes. Furthermore, chemokines recruiting a single class of effector cells can be biphasic, so that the emigration of these cells can be prolonged. For example, Salmonella invasion of cultured colonic epithelial cell monolayers induces maximal expression of IL-8, which selectively recruits and activates neutrophils, at four to six hours, while epithelial neutrophil activating protein-78 is maximally expressed 18-24 hours after bacterial invasion.[4,5] IL-6 secreted by epithelial cells can promote the transition from acute to chronic inflammation by altering chemokine profiles to favor monocyte recruitment.[6] This sequential activation of chemokines leads to progressive recruitment of effector cells with distinct functions, and also prolongs chemotactic signals to ensure efficient protective innate and acquired immune responses. In addition to chemotactic signals, activated NF-κB and MAP kinases in epithelial cells stimulate expression of adhesion molecules, including ICAM-1, MHC Class II molecules, cyclooxygenase-2 (COX-2), CARD15/NOD2 and certain proinflammatory cytokines, including IL-6.[7-10] These molecules expand the immunologic function of mucosal epithelial cells beyond being merely a sensor of injury to that of actively participating in the inflammatory response.[8] Mucosal defenses are further mediated by epithelial cell secretion of antimicrobial peptides, including α and β defensins, bacterial permeability-inducing peptide, lysozyme and mucins. Proinflammatory cytokines stimulate expression of β defensins and secretion of mucus.[11]

Macrophages

Resident mucosal macrophages, which comprise 8-15% of intestinal lamina propria mononuclear cells, are less responsive to bacterial adjuvants and inflammatory signals than are newly recruited monocytes due to low membrane expression of CD14 (LPS binding cofactor), CD11b (complement receptor 3) and CD16 (FcγIII receptor).[12-15] This relative non-responsiveness provides one protective mechanism preventing pathogenic responses to luminal commensal bacteria and accounts for the observation that the majority of cytokines produced in active IBD are derived from freshly emigrated immature macrophages.[13] Chemotactic signals that selectively stimulate monocyte migration include MIP-1α and β, MCP-1 and MCP-2.[16] Macrophages are scattered throughout the lamina propria and are preferentially positioned under the subepithelial basement membrane, where they can easily respond to invading pathogens and translocating microbial products. Bacterial components primarily induce expression of proinflammatory molecules (IL-1, TNF, IL-6) and chemokines. In addition, mucosal macrophages secrete abundant IL-10 in response to bacterial stimulants and proinflammatory molecules, serving to downregulate the inflammatory response. The importance in macrophages and neutrophils in inducing and maintaining intestinal inflammation is demonstrated by therapeutic responses of ulcerative colitis patients to granulocyte and monocyte aphoresis, leading to decreased mucosal production of TNF, IL-1, IL-6 and IL-8.[17,18] In addition, elimination of mucosal macrophages with rectal microspheres attenuates experimental colitis.[19]

Dendritic cells

Dendritic cells are efficient mucosal antigen presenting cells due to their avid uptake of soluble antigens, efficient antigen processing and presentation, full complement of costimulatory molecules and secretion of IL-12, IL-18 and IL-23 upon exposure to microbial adjuvants. Different types of dendritic cells (myeloid, lymphoid and plasmacytoid) occupy various regions of organized mucosal lymphoid follicles[20] in the resting state, while myeloid dendritic cells are dramatically increased during mucosal inflammation.[21] Migration of these cells to inflamed mucosal surfaces is governed by CCL 20 (MIP-3α) and CCL 9 (MIP-1γ), the expression of which is upregulated during inflammation, and perhaps β7 integrin.[21,22] M-DC8$^+$ monocyte precursors are preferentially located in subepithelial areas of Peyer's patches and secrete large amounts of TNF; these are increased in Crohn's disease and decreased by corticosteroids.[23] Maturation of dendritic cells is stimulated by exposure to bacterial microbial adjuvants, that signal through TLR membrane receptors, NOD intracellular receptors and NF-κB, while MHC II expression is stimulated by IFN-γ exposure as well as by NF-κB activation by bacterial adjuvants, IL-1 and TNF. Co-stimulatory molecules in mucosal antigen presenting cells are also upregulated by IL-1, TNF and microbial adjuvants.[24]

Mast cells

Mast cells comprise 2-5% of intestinal lamina propria mononuclear cells and are located in all layers of the intestine and stomach. Mucosal mast cells are preferentially located adjacent to neuronal synapses[25] and are activated by substance P, other neuropeptides, IgE binding to the IgE Fc receptor, activated complement, IL-3 and stem cell factor (C-kit).[26] Stimulated mast cells primarily secrete preformed granule constituents, such as histamine, serotonin and proteases, but also produce proinflammatory cytokines and arachidonic acid metabolites. Distinctive mucosal and connective tissue mast cell subsets in rats arise from common precursors but secrete different products.

Eosinophils

Mucosal eosinophils primarily secrete arachidonic acid metabolites and preformed biologically active molecules stored in granules, including eosinophilic cationic protein, major basic protein and peroxidase, but also can produce proinflammatory cytokines. These cells are activated by exposure to IgE, and to a lesser extent IgA and IgG, and are attracted to mucosal surfaces by eotaxin, MCP-3 and IL-8. The C-C chemokine eotaxin is selectively upregulated in intestinal epithelial cells by IL-4, but not by IFN-γ,[27] with contribution by IL-5.[28] In addition, ENA-78/CXCL5, IL-8/CXCL8 and TNF production by eosinophils is induced by TNF, but decreased by IFN-γ.[29] Eosinophils contribute to clearance of helminths, gastrointestinal injury and nerve damage in T_H2-mediated hypersensitivity reactions.[30]

Natural killer (NK) cells

NK cells are large granular lymphocytes that can express CD2 and αα homochimeric CD8, respond to IL-2, and produce IFN-γ, but do not express T cell receptors or surface immunoglobulin. Instead, they can coexpress myeloid markers such as CD11b and c, CD14 and the Fc receptor for IgG. Cytotoxic effects are largely independent of proinflammatory cytokine activity.

Mesenchymal cells

In addition to secretion of matrix components, including collagen and fibronectin that mediate fibrosis, and matrix metalloproteases that are involved in tissue remodeling and activation of latent TGF-β, mucosal fibroblasts, myofibroblasts and smooth muscle cells are active participants in innate immune responses. These cells can be activated by proinflammatory cytokines, bacterial products and TGF-β to proliferate, migrate and secrete IL-1, TNF, IL-6, IL-8, M-CSF, GM-CSF, metalloproteases, and express bioactive CD40 and adhesion molecules.[31-35]

Endothelial cells

Likewise, endothelial cells respond to proinflammatory molecules and bacterial products by expressing adhesion molecules such as ICAM-1, ECAM-1 (etc, etc) involved in the initial phase of cellular recruitment into an inflammatory focus, but also actively participate in inflammation by expressing proinflammatory cytokines, including IL-1, IL-6 and TNF, and chemokines.[36]

Mechanisms of stimulating cytokine expression

Introduction

Innate immune cells can be activated by a variety of mechanisms, including bacterial products and inflammatory mediators, such as IL-1, TNF, IL-6, neuropeptides and reactive oxygen metabolites. However, these diverse stimuli induce cytokine transcription through two dominant intracellular kinase signaling cascades, NF-κB and MAP kinases, thereby activating similar patterns of cytokine expression (figure 1). Different bacterial components selectively bind to various pattern recognition receptors to trigger NF-κB and MAPK.[37] Bacterial peptidoglycan, mycobacterial lipoarabinomannan, mycoplasma lipopeptides and heat shock protein 60 (HSP 60) bind to TLR-2, which can complex with TLR-3 or 6, viral DNA binds TLR-3, LPS selectively ligates TLR-4, flagellin binds to TLR-5, and the bacterial DNA motif CpG ligates TLR-9. Intracellular bacterial recognition is accomplished by CARD4/NOD 1 (ligates diaminopimelic acid), which is constitutively expressed in most intestinal cells, including epithelial cells, and by CARD15/NOD-2 (binds muramyl dipeptide), which is constitutively expressed in monocytes, dendritic cells and Paneth cells and is induced in intestinal epithelial cells by IFN-γ and TNF.[38-41]

The complexity of this system is increased by the ability of various TLR moieties to interact and by the ability of proinflammatory cytokines to affect expression of TLR and NOD molecules. For example, TLR-2 complexes with homologous TLR-1 to bind mycobacterial lipoarabinomannan and mycoplasma triacylated lipopeptides, while mycoplasma diacylated lipopeptides bind to TLR-2/TLR-6 complexes.[42,43] These combinatorial repertoires are postulated to enhance the ability of the host to discriminate diverse bacterial products.[44] In addition, efficacy of LPS binding to TLR-4 is markedly enhanced by co-ligation of LPS binding protein and CD14. As mentioned in earlier, intestinal macrophages have lower expression of CD14 than do monocytes, providing a mechanism for relative non-responsiveness to LPS produced by commensal enteric bacteria. Likewise, TLR and CARD15/NOD2 expression by native intestinal epithelial cells is relatively low, although Haller et al have demonstrated that *B. vulgatus* can stimulate expression of IL-6 and activate NF-κB in colonic epithelial cells after *in vivo* luminal colonization via LPS binding to TLR-4.[45,46]

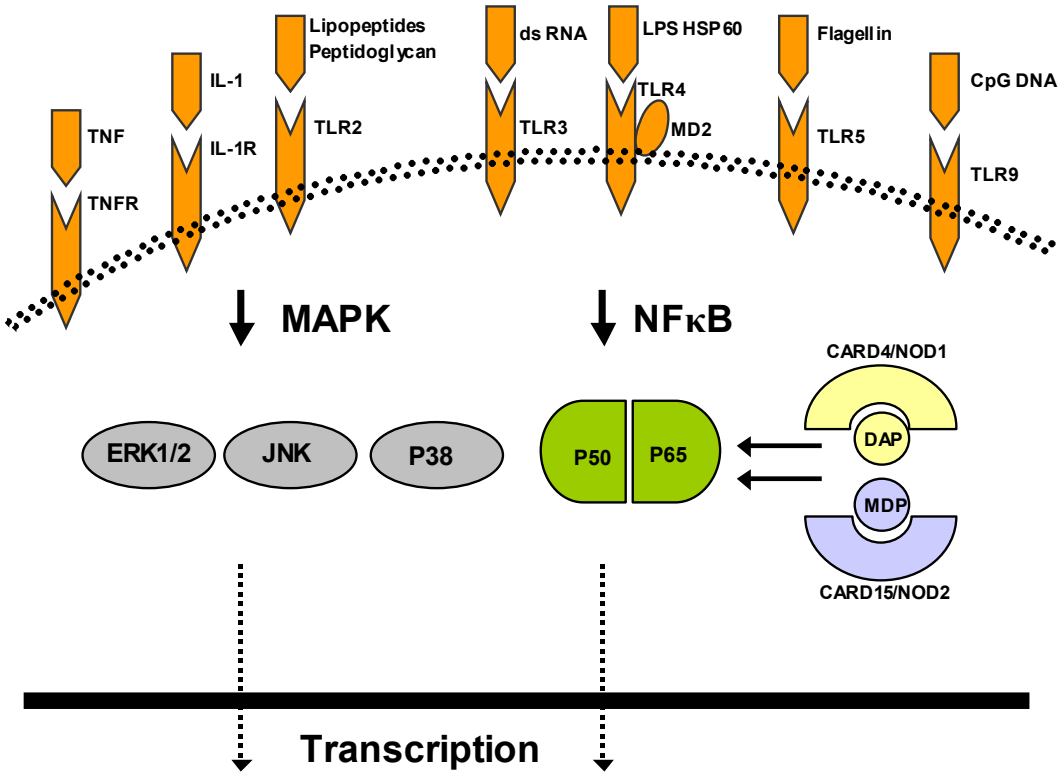

Figure 1. Activation of innate immune responses via ligation of pattern recognition receptors. A wide range of microbial adjuvants selectively bind to homologous membrane-bound TLR or to intracellular CARD/NOD receptors. These pattern recognition receptors, the IL-1 receptor (IL-1R) and tumor necrosis factor receptor (TNFR) signal through the central NF-κB and MAPK pathways to activate transcription of a large number of proinflammatory and protective molecules.

Similarly, feeding DNA from probiotic bacteria or *E. coli* can prevent and treat experimental colitis through interaction with TLR-9.[47] TLR-2 is expressed in human alveolar type II epithelial cells and macrophagesn.[48] IFN-γ upregulates expression, with resultant enhanced function, of CARD4/NOD1 and the TLR-4/MD-2 complex on intestinal epithelial cells[49,50] while TNF stimulates CARD15/NOD2 expression in epithelial cells.[9,10]

Once ligation of various TLR molecules and bacterial products occurs, signaling converges on central pathways that are shared proximally by the IL-1/IL-1 receptor (IL-1R) and more distally by the CARD/NOD and TNF signaling pathways (figure 2). The TLR and IL-1 signaling pathways require activation of toll-IL-1-1R (TIR) domain adaptor proteins, including MyD88, TIRAP and TRIF. Although transcription of most proinflammatory cytokines requires MyD88, ligation of TLR-3 and 4 can proceed through a MyD88-independent pathway mediated by TRIF. A TRIF-related adapter protein (TRAM) is required for TLR-4-specific induction of IFN-β, but not TLR-3 stimulated responses.[51] MyD88 dependent and independent TLR, IL-1β, TNF and CARD/NOD pathways activate NF-κB through activation of the IKK complex to phosphorylate IκBα, which is the ubiquinated and degraded by the 700 kDa proteasome complex, thereby freeing the previously complexed NF-κB heterodimer to be phosphorylated and translocate to the nucleus.[8] Once NF-κB has homed to the nucleus, it stimulates transcription of multiple cytokines by binding to κB regulatory elements on the promoter region of the cytokine genes (figure 1). In addition to transcriptional regulation by NF-κB and other relevant

transcription factors (figure 1), production and secretion of proinflammatory cytokines is dependent on stabilization of mRNA, protein translation, post-translational modification and in some cases, such as IL-1 and IL-18, processing by precursor molecules.

TNF receptor ligation will also activate NF-κB, but uses a different set of post-receptor scaffolding proteins (including TRAF6; figure 2). Similarly, CARD4/NOD1 and CARD15/NOD2, which selectively bind a diaminopimelic acid tripeptide from Gram-negative bacteria and muramyl dipeptide, respectively[41] activate NF-κB through the intermediate kinase Rip2 (RICK/CARDIAK), which appears to contribute to TLR, IL-1 and IL-18 signaling as well.[52] Of considerable interest to the pathogenesis of Crohn's disease, the three most common polymorphisms of CARD15/NOD 2, which are in the leucin rich repeat region that binds bacterial cell wall peptides, diminish NF-κB activation.[53]

Both IL-1, TNF and TLR activate parallel pathways in addition to NF-κB. A complex cascade of kinases lead to phosphorylation of c-jun NH2 terminal kinase (JNK) and p38 MAPK (figure 3). These kinases activate c-fos and c-jun, the heterodimers of the AP-1 transcription factor complex. AP-1 transcriptionally regulates most proinflammatory cytokines, many in conjunction with NF-κB.

Figure 2. Intracellular signal cascades activated by IL-1 receptor, TNF receptor or TLR ligation.

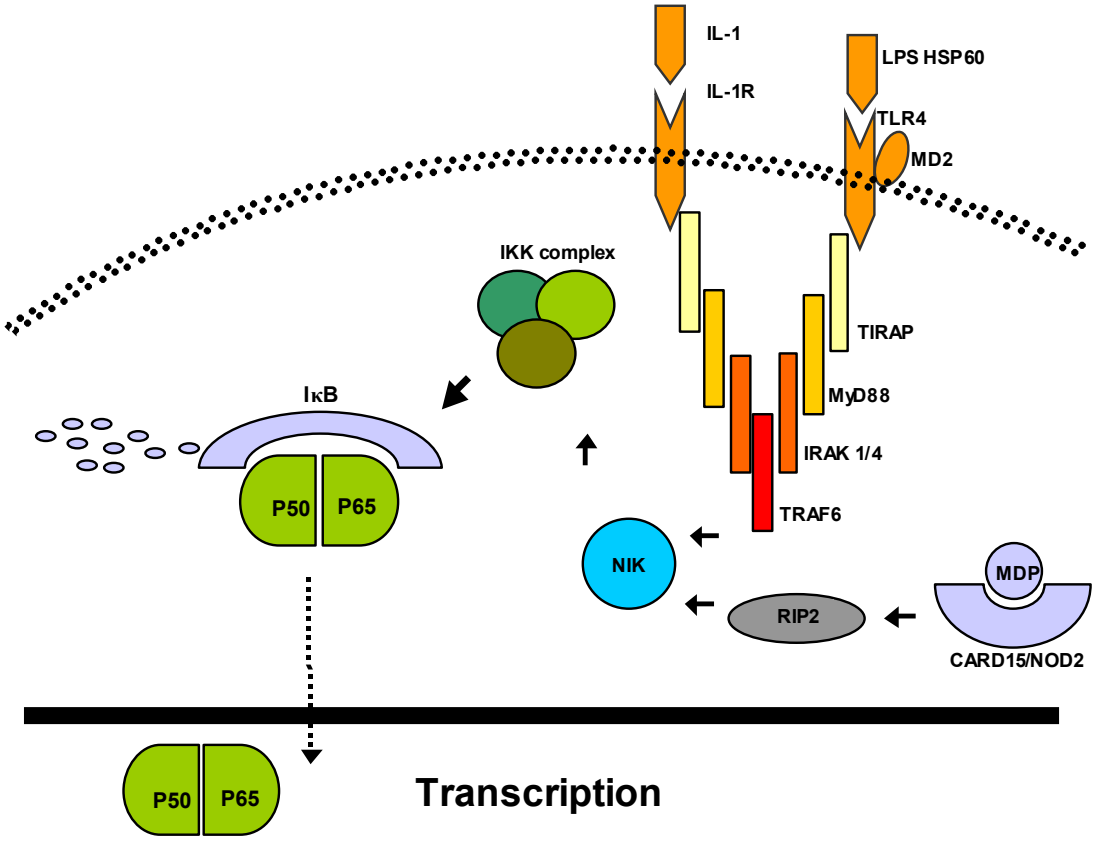

Figure 2A. Parallel signaling mechanisms of the IL-1R and TLR pathways. Ligation of IL-1 or LPS to IL-1R and TLR-4 respectively, activates signals through parallel signaling pathways involving a series of adaptor and scaffolding proteins and kinases. Activated kinases ultimately lead to the phosphorylation and degradation of IκBα. This releases NF-κB, which is phosphorylated and translocates to the nucleus, where it initiates transcription of a number of effector and regulatory molecules.

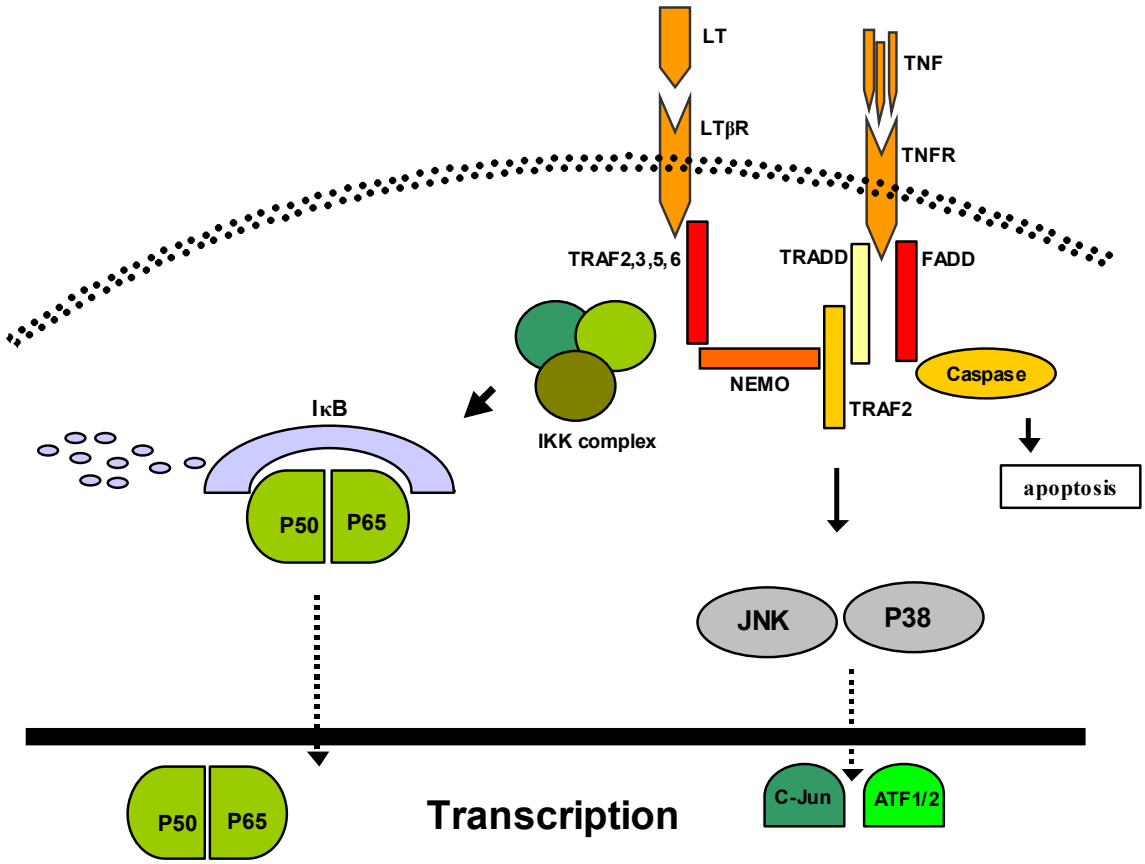

Figure 2B. Activation of multiple signaling and apoptotic pathways by TNF family member ligands. TNF (TNFα) or lymphotoxin (TNFβ) bind to specific receptors that can activate either the NF-κB or MAPK signaling cascades through a series of specific adaptor molecules. In addition, TNF can initiate apoptosis through death domain adaptor molecules and caspases.

Of considerable relevance to bacterial homeostasis, response to infection and treatment of inflammation, selective blockade of JNK and p38 MAPK can have different effects, as suggested by their independent activation, function and regulation. JNK, p38 MAPK, and extracellular signal-regulated protein kinase (ERK 1/2) are phosphorylated in active IBD,[54,55] and an uncontrolled study with an inhibitor of both p38 MAPK and JNK impressively inhibits active Crohn's disease.[56] However, low dose LPS administration to human volunteers selectively activated p38 MAPK but not JNK in PBMNC.[54] Furthermore, a selective p38 MAPK inhibitor caused increased weight loss and higher levels of colonic cell TNF production in a TNBS model of colitis, although it decreased TNF production (S. van Deventer, unpublished observations). Thus, these pathways must be considered separately in mucosal inflammation.

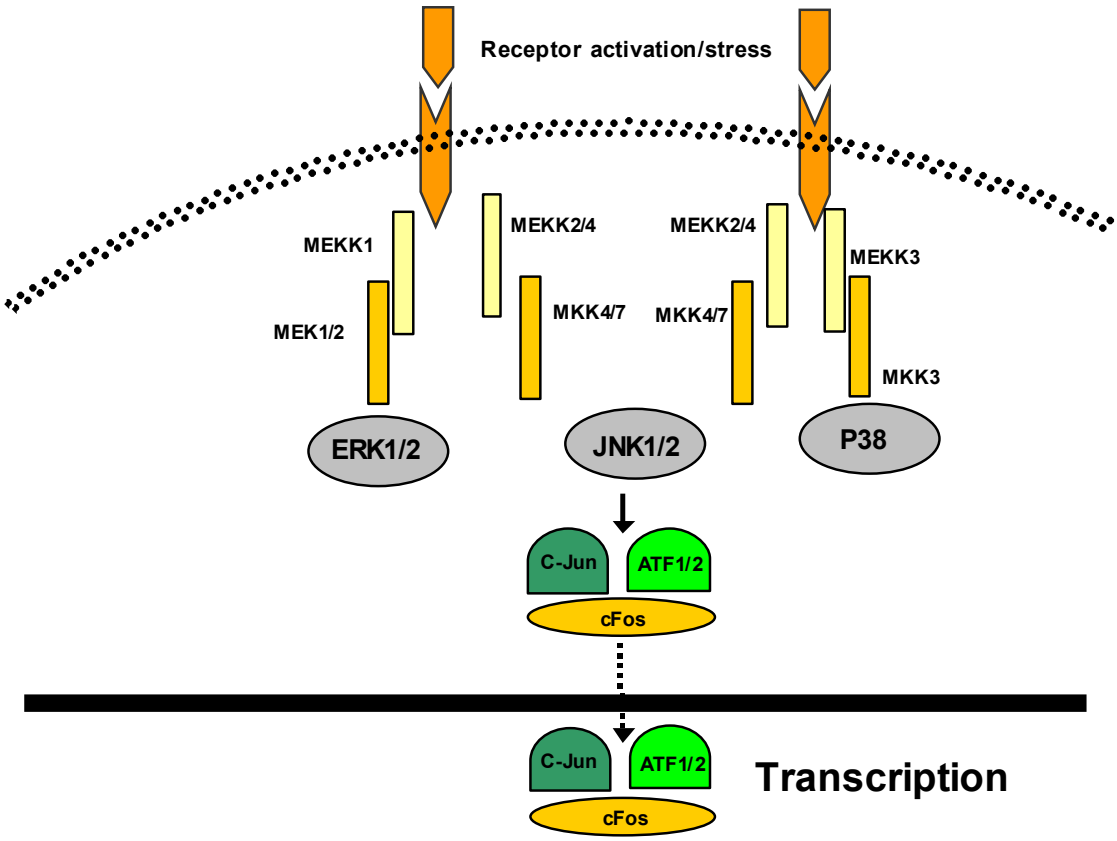

Figure 3. Activation of MAP kinases by multiple stimuli. A variety of stimuli, including growth factors, hormones and proinflammatory cytokines (TNF, IL-1, etc) can activate a series of kinases that lead to liberation of transcription factors that operate in parallel with NF-κB. the MAPK effector molecules c-Jun and c-Fos can heterodimerize to form AP-1, or these molecules and ATF 1/2 can bind to DNA regulatory elements alone or in various combinations to regulate gene transcription.

IP3/AKT stimulates intracellular calcium fluxes, but also mediates calcium/calmodulin-dependent protein kinases kinase suppression of IL-1 and TLR signaling by uncoupling IRAK-1 and MyD88 activity. In addition, TNF inhibits AKT phosphorylation, which is decreased in portal hypertensive gastropathy. Inhibition of AKT is postulated to contribute to gastric epithelial injury, since AKT stimulates epithelial cell proliferation, migration and survival. Anti-TNF treatment restored gastric AKT levels and induced healing.[58] IL-1, TNF and IL-6 activate NF IL-6, a transcription factor binding along with NF-κB and AP-1 to the promoters of IL-6 and IL-8 to positively regulate transcription of these cytokines.[59] Finally, IL-1β can stimulate the ERK 1/2 and AKT pathways via the association of phosphorylated SHPS and SHP-2.[60] LPS or LPS plus IFN-γ stimulation of dendritic cells increases ERK and p38 MAPK while stimulating IL-10 and IL-12. Blockade of ERK selectively decreases IL-10 and enhances IL-12 production, suggesting that ERK selectively induces IL-10, an endogenous inhibitor of IL-12.[61]

The regulation of these interacting pathways is extremely complex and has been evaluated predominantly in isolated cell lines and in PBMNC. It remains to be determined whether mucosal innate immune cells follow these paradigms or whether they exhibit unique regulatory features.

Mechanisms of inhibiting proinflammatory cytokine expression and function

Regulation of the expression and activity of proinflammatory cytokines is a key component of maintaining mucosal homeostasis and preventing excessive tissue injury after an inflammatory event. In addition, therapeutic blockade of innate immune responses is vital to treating mucosal inflammation.

Endogenous inhibitory mechanisms

Activation of signaling pathways by inflammatory cytokines or bacterial products stimulates a number of overlapping protective pathways that inhibit proinflammatory cytokine production by innate immune cells (table 2). Many of these protective mechanisms can be induced by multiple cytokines, while others are selectively induced by a single stimulant. For example, IL-1, TNF and IL-6 activate the hypothalamic/pituitary/adrenal (HPA) axis culminating in the release of immunosuppressive corticosteroids. The importance of this protective pathway is illustrated by the increased intensity and prolongation of experimental inflammation accompanied by increased IL-1 and TNF levels in adrenalectomized rats or following pharmacological blockade of corticotrophin releasing hormone.[62] In addition to the more widely recognized proinflammatory molecules induced by IL-1, TNF and bacterial molecules, NF-κB activation leads to increased expression of multiple protective genes, including IκBα, COX-2, inducible nitric oxide synthetase (iNOS) and PPARγ. Production of IκBα is important in deactivating NF-κB by complexing nuclear p65/p50 heterodimers in interrupting gene transcription and transporting this complex back to the cytoplasm where it exists in an inactive complexed form. Although PGE_2, PGI_2 and other prostaglandins released during inflammation enhance epithelial chloride secretion, increase vascular permeability and stimulate smooth muscle contractions thereby causing diarrhea, edema and hypermotility, these products also inhibit proinflammatory cytokine production[63] and stimulate mucosal healing.[64] PGJ_2 may mediate protective effects through PPARγ activation. The importance of prostaglandin production in mucosal homeostasis, resolution of inflammation, and downregulation of inflammatory cytokines is illustrated by small intestinal and colonic inflammation following two doses of indomethacin in susceptible rat strains,[65] piroxicam triggering colitis in IL-$10^{-/-}$ mice on a resistant (C57BL/6) background,[66] potentiation of DSS-induced colitis in COX-2 deficient mice,[67] and inhibition of DSS-induced colitis by ligation of EP4, the PGE_2 receptor.[68] In each of these models mucosal IL-1 and TNF are increased. Similarly, nitric oxide produced as a consequence of proinflammatory cytokine stimulation of iNOS has important protective properties. Nitric oxide inhibits NF-κB activation by blocking the IRAK/TRAF-6 interaction[69] and inhibits caspase 1/IL-1β converting enzyme (ICE) activity by 5-nitrosylation.[70] IL-10 is induced by a number of agents, including IL-1, TNF, IL-6, IL-12, IFN-γ and bacterial products. Of interest, the induction of IL-10 by bacterial products lags behind that of proinflammatory cytokines. Incubating unfractionated mesenteric lymph node cells or splenocytes with LPS induces maximum TNF production by 4 hours, IL-1 by 6-8 hours, and IL-12 by 24 hours, but IL-10 secretion reaches maximal concentrations only after 72 hours of stimulation.[71] This progressive induction of first proinflammatory and then protective cytokines suggests a compensatory regulatory mechanism to downregulate responses to an inflammatory stimulus. The importance of endogenous IL-10 in host protection to the aggressive luminal environment is dramatically illustrated by spontaneous colitis in IL-$10^{-/-}$ mice raised in the presence of commensal bacteria[72,73] and strikingly elevated IL-1β, TNF, IL-12 and IFN-γ secretion by LPS or

cecal bacterial lysates stimulated mesenteric lymph node cells in IL-10$^{-/-}$ versus wild-type mice.[71] Secretion of soluble receptors by activated cells can bind IL-1 and TNF, thereby neutralizing their biologic effects. Finally, a number of inflammatory stimuli, including IL-1β, can stimulate expression of IL-1 receptor antagonist (IL-1RA), which competitively inhibits IL-1 receptor binding and downstream signaling.[74] Of interest, IL-10 selectively inhibits IL-1β to a greater extent than IL-1RA in splenocytes exposed to LPS, profoundly altering the IL-1RA/IL-1 ratio to a less inflammatory profile.

Proinflammatory cytokines can selectively stimulate inhibitors that provide negative feedback on the inflammatory response. For example, TNF stimulates intracellular A20, which inhibits TNF signaling.[75] The importance of A20 in mucosal protection is evident by the colitis that develops in A20$^{-/-}$ mice.[75] Although not selective, the concept of an inflammatory pathway stimulating production of an inhibitor that suppresses functions of the inductive stimulus is well documented, with IL-1 stimulating IL-1RA and NF-κB increasing expression of IκBα being two obvious examples.[8,74]

Mechanisms by which endogenous inhibitory molecules exert their protective effects are still not entirely clear. Many immunosuppressive molecules have multiple modes of action and target central signaling pathways. For example, corticosteroids inhibit phospholipase A2, thereby blocking arachidonic acid liberation, blocks NF-κB activity, suppresses transcription by binding to the glucocorticoid regulatory element and the promoter of many proinflammatory molecules, prevents transcription factor binding to promoters by histone deacetylation, stabilizes lysomal membranes, and inhibits adhesion of effector cells to endothelial cells.[76] Similarly, IL-10 inhibits NF-κB by multiple mechanisms, yet to be fully understood. IL-10 transiently delays IκBα degradation after an inflammatory stimulus, but its probable mode of action is to inhibit p65 binding to DNA, although it does not block NF-κB p65/p50 nuclear transmigration.[77] In addition, IL-10 phosphorylates STAT3 and stimulates production of the inhibitory molecule SOCS3.[78,79] TGF-β regulates aggressive T cell function by suppressing lymphocyte proliferation and activation, but also stimulates IL-10 expression, perhaps explaining some of its diverse biologic activities.[80] TGF-β phosphorylates SMAD2 and 3, leading to activation of SMAD4. Phosphorylation of SMAD2 in colonic epithelial cells is evident 7 days after selective colonization of germ-free rats with *B. vulgatus*, which is temporally related to downregulation of *in vitro* epithelial NF-κB stimulation and IL-6 production.[46] In vitro studies showed that endogenous TGF-β could suppress LPS-induced IL-6 secretion by cultured colonic epithelial cells and affect NF-κB activation by blocking histone H3 acetylation.[46]

Exogenous compounds inhibiting proinflammatory cytokines

Although beyond the scope of this chapter, multiple clinically relevant pharmacological agents, including translational clinical comments as well as biologic agents, have proven abilities to suppress proinflammatory cytokine transcription and biologic activity (table 3). Therapeutic effects of these compounds, particularly those selectively active for individual cytokines, in clinical use and experimental intestinal inflammation confirm the key contribution of proinflammatory cytokines in acute and chronic mucosal inflammation. Examples of the beneficial activities of compounds with selective activities are provided in sections describing individual cytokines or signaling pathways.

Approach	Target	Mechanism
Corticosteroids	IL-1, 6, 12, TNF, chemokines	Inhibits NF-κB, cytokine transcription
IL-10 (recombinant bacteria, transfected T cells)	APC, IL-12, IL-1, TNF, IL-6, chemokines	Inhibits NF-κB, APC activation
Block TNF–monoclonal antibodies, binding proteins, receptors	TNF	Inactivate secreted TNF, induce apoptosis of cells with membrane–bound TNF
Thalidomide analogues	TNF, IL-1	Inhibit synthesis
Pentoxifylline	TNF, IL-1	Inhibit synthesis
NF-κB–p65 antisense oligonucleotides, proteosome inhibitor	IL-1, TNF, IL-6, IL-12, chemokines	Block transcription
MAPK inhibitors–p38 MAPK, JNK	IL-1, 6, 12, TNF, chemokines	Block transcription

Table 3. Therapeutic approaches to blocking inflammatory cytokines produced by activated innate immune cells.

Structure and function of individual proinflammatory cytokines

Consistent with their shared signaling pathways and receptors, many proinflammatory cytokines and chemokines have overlapping functions, yet each retains a unique pattern of responses. For example, IL-1 and TNF receptor ligation both stimulate NF-κB, MAP kinases and AKT, yet TNF has a greater ability to activate T_H1 lymphocyte responses and has an independent effect on apoptosis. Many chemokines share common heterodimeric molecules and receptors, yet recruit different cell populations. These differential properties may be due to selective production by different cells or a result of differential kinetics of production. Many cytokine responses are either autocrine or paracrine in nature, such that biologic activity depends on the local cellular milieu. In addition, the activation state of a target cell can profoundly affect its response to a given stimulus as a result of up or downregulation of membrane receptors, induction of endogenous protective molecules or the degree of phosphorylation or dephosphorylation of signaling molecules.

Because of shared inductive mechanisms, in large part via NF-κB and AP-1 signaling pathways, proinflammatory cytokines tend to be expressed in tandem. For example, IL-1β, TNF, IL-6 and various chemokines are all induced in active IBD,[2,81,82] while a characteristic profile of cytokines is induced by IL-1, TNF or invasive bacteria in cultured colonic epithelial cells.[7] Thus, it is physiologically and pathophysiologically important to consider interactive, additive and synergistic activities of cytokines rather than isolated expression and activities. However, the present discussion will artificially consider the biologic effects of individual cytokines. Although many of the examples supporting physiologic and pathophysiologic effects of these cytokines are

related to intestinal inflammation because of the wealth of investigations in this field and the particular expertise of the author, similar activities are found at other mucosal sites.

IL-1 family (IL-1α, IL-1β, IL-1RA and IL-1β converting enzyme)

Structure and secretion

IL-1 occurs in two forms, IL-1α and IL-1β, which share similar receptor binding properties, and hence overlapping function. IL-1α is membrane bound or intracellular, therefore mediates its effects by cell contact or following liberation following cell lysis. Pro IL-1α (31 kDa) is cleaved by calpain or by extracellular proteases to a 17 dDa form. In contrast, IL-1β is 6 as an 18 kDa precursor that lacks a leader sequence, therefore remains in an intracellular cytoplasmic location unless activated by cleavage of ICE (caspase 1).[83] ICE itself is constitutively expressed as a 45 kDa inactive precursor that is cleaved at two locations to form an active heterodimer. ICE is cleaved by various caspases, including ICE itself, following cellular activation by bacterial products, proinflammatory cytokines or CD40 ligation, but caspase activity is inhibited by nitric oxide. Following cleavage, biologically active IL-1β is secreted. IL-1RA is also present in two forms. A 22 kDa secreted form competitively inhibits IL-1 bioactivity by competitively binding to the two IL-1 receptor isoforms. A second splice variant form lacking a leader sequence remains in an intracellular cytoplasmic location.[84]

Expression

IL-1α and IL-1β are induced by multiple proinflammatory signals and are expressed in a variety of cell types. Levels are low in the normal intestine, but are dramatically increased during active inflammation.[81,85-89] Tissue staining and cell extraction demonstrate that lamina propria macrophages, particularly newly immigrated cells with monocyte features, account for the majority of IL-1 production, although mesenchymal cells, endothelial cells and to a lesser extent epithelial cells express mRNA and produce protein.[14,89,90] IL-1RA is constitutively expressed in the normal intestine and in increased amounts following inflammation. Intracellular IL-1RA is constitutively expressed in intestinal epithelial cells, with mRNA expression in the crypt and accumulation of protein with cellular maturation.[91] ICE is constitutively expressed, with expression but not cleavage upregulated by IFN-γ.

Signaling

IL-1α and IL-1β bind to two receptor isoforms that are selectively expressed on different cell types relevant to mucosal tissues. IL-1RI is present on intestinal epithelial cells, endothelial cells, mesenchymal cells and T lymphocytes, while IL-1RII is expressed on monocytes/macrophages, neutrophils and B cells. IL-1RII binds IL-1 but does not transduce signals, raising the concept of a secreted "decoy" receptor that can inhibit responses.[92] Ligation of either receptor transduces signals through a complex of kinases and scaffolding proteins (IRAK-1, TRAF-6, TAK1/TAB and Rip 2) to activate the NF-κB pathways (figure 2). A comprehensive study by Mifflin et al shows that IL-1α stimulates COX-2 mRNA transcription in intestinal myofibroblasts by activating the NF-κB, ERK, and protein kinase C (PKC) pathways.[31] In parallel, IL-1α-stimulated p38 MAPK stabilizes COX-2 mRNA.

Function

Activation of NF-κB, MAPK and AKT by ligation of the IL-1 receptors by IL-1α or β increases expression of a number of molecules involved in the inflammatory response (figure 1). Induction of IL-1, TNF, IL-6, IL-12 and chemokines by lamina propria innate immune cells and

mesenchymal cells amplify the inflammatory response, while stimulation of chemokine secretion and expression of adhesion molecules on mucosal epithelial cells leads to the emigration, transmigration and excretion of effector cells.[82] IL-1 stimulates expression of costimulatory molecules on antigen presenting cells and upregulates IL-12 secretion, thereby enhancing T cell activation. Induction of metalloproteases results in matrix destruction, ulceration and possibly fistula formation. Finally, enhanced expression of COX-2 leads to rapid production of eicosanoids that mediate epithelial chloride secretion and block sodium absorption, leading to diarrhea and bronchial secretion, smooth muscle contractions resulting in cramps, airway spasm and diarrhea, and increased vascular permeability. Induction of iNOS, with resultant nitric oxide synthesis, enhances intracellular killing of phagocytosed microbial agents, inhibits smooth muscle contractions, possibly leading to ileus associated with intestinal inflammation, and regulates mucosal blood flow. Finally, IL-1 mediates anorexia associated with experimental colitis through induction of 5-HT in the hypothalamus.[93] In addition to proinflammatory effects, IL-1 stimulates production of anti-inflammatory molecules such as prostaglandins, nitric oxide and IL-1RA that inhibit inflammation. For example, IL-1β stimulates production of PGE_2, 6-keto PGI_2 and thromboxane B2 in the rabbit colon[94] and administration of IL-1β 24 hours before administration of formalin and immune complexes in rabbits attenuates colitis through stimulation of endogenous prostaglandins.[95]

The net proinflammatory properties of endogenous IL-1 are documented by the ability of recombinant IL-1RA to attenuate experimental colitis in a number of models.[85,86,96] Administration of IL-1RA not only decreases inflammation, but also inhibits expression of a number of proinflammatory cytokines by the inflamed colon.[86] A trial of subcutaneous rIL-1RA in ulcerative colitis was not completed. Local mucosal administration via engineered bacteria may prove to be a better approach than systemic administration. Colonization of IL-$2^{-/-}$ mice with IL-1RA-secreting *Streptococcus gordinii* enhanced weight gain, although the colon was not examined.[97] Attenuation of acute and chronic colitis in $ICE^{-/-}$ mice[98] and the ability secreted IL-1 to predict relapse of inactive Crohn's disease provide additional evidence of a pathogenic role of IL-1 (and IL-18).[99]

Bioactivity of IL-1α and IL-1β is dependent on ligation of either IL-1RI or RII. Thus, the balance of IL-1 and IL-1RA determines receptor activation. Although the levels of IL-1RA are increased and the IL-1/IL-1RA ratio is decreased during intestinal inflammation due to increase of IL-1 to a greater degree than IL-1RA.[81,86,87,100] This abnormal ratio may be a secondary phenomenon since the ratio correlated with disease activity of IBD and was also decreased in nonspecific mucosal inflammation.[86,100] However, an etiologic role for defective IL-1RA activity is suggested by the association of polymorphisms in allele 2 of IL-1RA with ulcerative colitis and pouchitis by some, but not all authors.[101-104]

TNF and lymphotoxin

Structure and secretion

TNF (TNFα, cachexin) is a 17 kDa protein that is produced as a 26 kDa precursor (pro-TNF). Pro-TNF is cleaved by TNFα cleavage enzyme (TACE or ADAM-17) or ADAM-10, which are zinc binding metalloprotease disintegrin members of the adamalysin family.[105,106] Secreted TNF forms trimeric complexes that optimally bind receptors. Structurally related lymphotoxin (TNFβ, LTa) is also secreted as a trimer, but can also complex with LTβ to form a membrane-bound heterodimer that has distinct functions. The TNF family of ligands and receptors comprise a complex and rapidly evolving group of immunologically relevant proteins

and costimulatory molecules that include TNF, LTα, LTα/β, Light, FasL, CD40L, TRAIL, RANKL, 4-1BBL, OC40L, GITRL and BAFF.[107]

Expression

Numerous cells in the inflamed mucosa produce TNF, including macrophages, T_H1 lymphocytes, mast cells, mesenchymal cells, endothelial cells and even epithelial cells, with activated macrophages being the primary source. Constitutive expression in the noninflamed gut is low, but is dramatically increased during infection or inflammation in response to NF-κB and MAPK activation by bacterial products and IL-1. Tissue levels of TNF mRNA and protein in IBD have been somewhat controversial, with some investigators showing increases in Crohn's disease tissue and stool that correlates with disease activity and others finding no consistent elevation.[81,108-110] One reason for these inconsistent results is the relative instability of TNF mRNA, which is dependent on the Au-rich elements (ARE) in the 3 prime untranslated region of the gene. Deletion of this ARE region leads to increased circulating and intestinal levels of TNF.[111] TNF is primarily produced by lamina propria macrophages, but is also expressed in subepithelial dendritic cell precursors ands activated T_H1 cells.[23,108]

LTα is expressed in the same cell populations as TNF and is also increased in inflamed mucosal tissues. LTα secretion by colonic biopsy explants is increased in Crohn's disease relative to ulcerative colitis.[112] LTβ is more selectively expressed by lymphoid cells.

Signaling

TNF binds to two membrane bound receptors, TNFRI (p55) and TNFRII (p75), that are expressed in epithelial, bone marrow derived, mesenchymal and endothelial cells at mucosal surfaces.[113] Expression of TNFRII is increased in colonic epithelial cells during experimental colitis and active IBD.[114] Ligation of TNFRII by TNF or LTα trimers results in activation of proinflammatory effects mediated by stimulation of NF-κB, MAPK, PKC and AKT (figure 2). Ligation of TNFRI by TNF or LTα selectively induces apoptosis through a series of death domains (Fas-activated death domain, FADD, etc). The TNF receptor-associated death domain (TRADD) is common to both pathways, which then diverge along two pathways regulated by TNF receptor-associated factor (TRAF) isoforms. TRAF 1 and 2 stimulate NF-κB and MAPK, while FADD mediates apoptotic signals through caspases. The α/β complex and Light bind to the LTβR.

Function

TNF stimulates both inflammatory responses in a variety of mucosal cell populations and induces apoptosis of epithelial cells. The ability of TNF to induce apoptosis is augmented by synergistic activities with IFN-γ.[115,116] The inflammatory effects are quite similar to those of IL-1, since both molecules activate NF-κB, MAPK and AKT (figure 1), although cellular targets may vary due to differential expression of IL-1 and TNF receptors on different cell populations. The expression of TNFRII on epithelial cells may relate to mechanisms of mucosal hyperplasia during inflammation.[114] Transfer of $CD4^+$ $CD45RB^{hi}$ T cells from wild-type and $TNF^{-/-}$ mice into wild-type or $TNF^{-/-}$ $RAG2^{-/-}$ mice demonstrates the importance of TNF production by non-T cells.[117] Like IL-1, TNF stimulates production of a variety of proinflammatory cytokines, chemokines, adhesion molecules, costimulatory molecules, COX-2, iNOS and secreted matrix metalloproteases. In addition, TNF activates osteoclasts and inhibits osteoblast function, leading to bone resorption. Furthermore, TNF stimulates activity of lipoprotein lipase, decreases appetite at the hypothalamic level, leading to weight loss, hence its original name cachexin. Systemic

injection of TNF induces mid small bowel inflammation and necrosis, possibly mediated through induction of platelet activating factor[118] and ΔARE transgenic mice overexpressing TNF spontaneously develop ileal inflammation, arthritis and eventually multiorgan failure.[111] Ileal inflammation in this model depends on the presence of both TNFRI and II, $CD8^+$ T cells, IFN-γ and IL-12 and is mediated by either myeloid or T cell-derived TNF.[119] TNF neutralization attenuates inflammation in a variety of experimental colitis models[120-122] as well as ileal disease in the SAMP 1/Yit T cell transfer model.[123] However, in these models blockade of endogenous TNF was not as effective as inhibition of IL-12 in preventing or reversing intestinal inflammation. However, a chimeric IgG 1 antibody to TNF (infliximab) has dramatic results in Crohn's disease, inducing responses in 65% of patients with luminal inflammation[124] and 68% of patients with fistulae.[125] However, these impressive results appear to be due to induction of apoptosis of activated T cells and monocytes bearing membrane-bound TNF rather than neutralizing free TNF since etanercept, a fusion protein of the extracellular domain of the p75 TNF receptor and the Fc portion of IgG, is not effective in Crohn's disease, despite equal binding affinity to TNF as infliximab.[126-128] Whether the decreased efficacy of other more fully humanized anti-TNF antibodies in Crohn's disease relates to decreased induction of apoptosis remains to be determined,[129] and will determine the effectiveness of PEGylated antibodies undergoing trials in Crohn's disease. Finally, thalidomide, a TNF synthesis inhibitor, is effective in Crohn's disease, but well documented toxicities preclude its widespread use.[130,131] The effectiveness of infliximab in ulcerative colitis is being evaluated by a large trial to resolve divergent early results.[132,133] TNF is an important mediator of intracellular bacterial killing through upregulation of iNOS. Blockade of TNF is associated with increased bacterial infections (abscesses, sepsis) as well as reactivation and dissemination of mycobacterial infections.[127] In an intestinal xenograft model of amebic colitis, TNF blockade was more effective than IL-1R blockade in decreasing explant inflammation, although *E. histolytica* invasion was unchanged.[121]

Animal models demonstrate an important function of LTα/β in mucosal immune development and inflammation. LTα/β, with IL-7, regulates lymphoid tissue development, notably Peyer's patches and mesenteric lymph nodes, during embryogenesis and maintains secondary lymphoid structure in adults.[134] In addition, LTβ R signaling is required for formation and maintenance of follicular dendritic cell networks. More vigorous colitis in mice deficient in organized lymphoid tissue following neonatal blockade of LTβ R suggests that cells in these tissues are protective.[135] In contrast, inhibition of LTα/β and Light activity by soluble LTβ R Ig fusion protein attenuated T cell-mediated colitis as effectively as anti-TNF antibody.[136] The latter results suggest that both LTα/β and TNF mediate chronic colitis.

IL-6, soluble IL-6 receptor (IL-6R and gp130)

Structure and secretion

IL-6 is secreted without processing. Its receptor complex consists of two distinct membrane-bound glycoproteins: an 80 kDa cognate receptor (IL-6R) and a 130 kDa signal transducing element (gp130). Membrane bound IL-6R can be shed after proteolytic cleavage by bacterial-derived metalloproteases and a yet to be fully defined mammalian metalloprotease distinct from TACE.[137] An alternative mechanism is differential mRNA splicing leading to an isoform that lacks membrane-spanning and cytoplasmic domains.[137]

Expression

IL-6 is produced by a variety of cells at mucosal surfaces, including macrophages, epithelial cells, mesenchymal and endothelial cells. The normal intestine has detectable amounts

of IL-6, which is dramatically upregulated in the inflamed intestine of patients with Crohn's disease, ulcerative colitis and nonspecific inflammation, as well as during experimental colitis, with tissue levels correlating with the degree of inflammation.[138-143] Similarly, circulating levels of IL-6 and sIL-6R correspond with the activity of intestinal inflammation and have been proposed as indicators of disease activity.[144,145] IL-6 expression is induced by IL-1, TNF (through NF-κB and MAPK), IFN-γ (through STAT-1), LPS (through NF-κB), TGF-β, heat shock proteins and prostaglandins.[46,146,147] IL-1β induces expression of IL-6 through transient activation of NF-κB; this is potentiated by IFN-γ through prolongation of NF-κB activation.[147] Polymorphisms of the IL-6 gene are not associated with Crohn's disease.[148]

Gp 130 is constitutively expressed on most cells, while membrane bound IL-6R is selectively expressed on monocytes, neutrophils, T and B lymphocytes and hepatocytes. sIL-6R shedding by monocytic cell lines is enhanced by exposure to phorbol esters and certain bacterial toxins, but not IL-1β, TNF, IFN-γ, IL-4, IL-6, IL-10, chemokines or growth factors.[149] However, neutrophils shed sIL-6R when exposed to C-reactive protein (CRP) through ligation of the Fcγ RIIa.[150]

Signaling

IL-6 can transduce signals in two ways (figure 4).[137] IL-6 ligation of membrane-bound IL-6R induces homodimerization of membrane bound gp130. Stimulation of this complex activates gp130-associated cytoplasmic tyrosine kinases (JAK1, JAK2 and Tyk2), which phosphorylate STAT3. An alternative mechanism of activating cells that express only gp130 is trans-signaling, which is mediated by ligation of sIL-6R–IL-6 complexes with membrane bound gp130.[151] Trans-signaling then causes homodimerization of membrane gp130 and identical intracellular phosphorylation of STAT3. In parallel, either mechanism of signaling activates AP-1 and NF-IL-6 through the Ras/Raf pathway. A mechanism of regulating this pathology is provided by secretion of soluble gp130, which when bound to IL-6/sIL-6R prevents trans-signaling. IL-11 also transduces signals through gp130.

Function

IL-6 has pleiotropic effects on many cells, with both pro- and anti-inflammatory properties. IL-6 can promote mucosal inflammation by inducing proliferation, activation and prevention of apoptosis of T lymphocytes, differentiation of B cells and stimulation of immunoglobulin production. At the same time, this molecule promotes neutrophil clearance from inflammatory foci,[147] decreases neutrophil chemokine production, activates the acute phase response (hepatic synthesis of CRP, serum amyloid A and haploglobulin), and induces expression of IL-1RA and the soluble p55 TNFR.[137] IL-6 mediates IFN-γ-induced neutrophil clearance by decreasing KC production and enhancing PMN apoptosis through induction of caspase 3.[147] IL-6 also promotes the transition from acute to chronic inflammation by recruitment of monocytes by altering chemokine profiles.[6] In contrast, trans-activation of T cells through ligation of the sIL-6R/IL-6 complex with membrane gp130 inhibits apoptosis of activated T cells from Crohn's disease patients and mice with T_H1-mediated colitis through induction of the anti apoptotic genes bcl-2 and bcl-xl.[103,151]

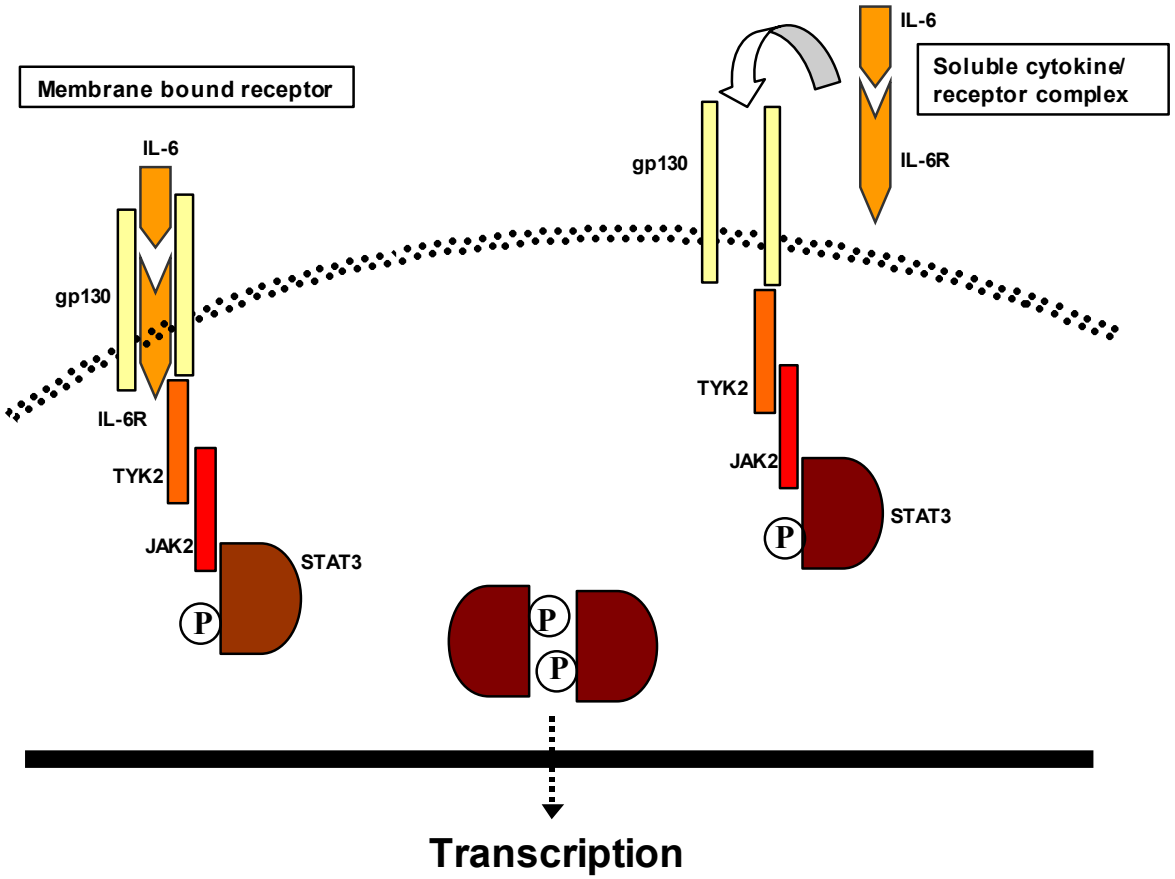

Figure 4. IL-6 signaling pathways. IL-6 can signal by 2 pathways. Ligation of membrane-bound IL-6 receptor (IL-6R) causes homodimerization of membrane-bound gp130. This complex activates tyrosine kinases that phosphorylate STAT3, which homodimerizes to activate AP-1 or NF-IL-6. These transcription factors activate expression of a number of proinflammatory genes. An alternative transsignaling pathway is mediated by complexing secreted IL-6 with soluble IL-6 receptor (sIL-6R), which then binds to membrane-bound gp130. Subsequent signaling mechanisms are identical to ligation of membrane-bound receptor.

A net proinflammatory effect of the trans-activating properties of experimental T_H1-mediated colitis is demonstrated by attenuation of disease by blocking sIL-1R-mediated signaling by anti-IL-6R antibodies or a gp130-Fc fusion protein.[151-154] However, IL-$6^{-/-}$ mice exhibit increased expression of TNF, IFN-γ, GM-CSF and MIP-2, as well as neutrophilia during acute inflammation.[155] Thus it is probable that IL-6 stimulates both protective and inflammatory pathways in intestinal inflammation, perhaps by downregulating acute innate-mediated effects and stimulating T cell-mediated events. A primary role for circulating IL-6 in bone resorption is suggested by antibody blockade experiments using serum from patients with active Crohn's disease.[156]

IL-12 and IL-23

These immunoregulatory cytokines that polarize T_H1 immune responses are briefly mentioned since they are produced by activated innate immune cells in response to many of the same stimuli that activate proinflammatory cytokines.

Structure

IL-12 is a homodimeric 70 kDa protein composed of p40 and p35 subsets. IL-12 p40 can also engage p19 to form IL-23, which has many functions overlapping with IL-12.

Expression

IL-12 is produced primarily by activated antigen-presenting cells, especially dendritic cells as well as activated macrophages. Although noninflamed $CD8^+$ lymphoid dendritic cells produce IL-12 in preference to IL-10 and CD11b+ myeloid dendritic cells have very little IL-12 production but high levels of IL-10 in the normal intestine.[157] IL-12 production by mucosal CD11c+ dendritic cells and CD11b+ cells (macrophages and myeloid dendritic cells) is dramatically increased during T_H1-mediated colitis in myeloid STAT3 conditional knockout mice.[158] Likewise, IL-$10^{-/-}$ myeloid dendritic cells stimulated with LPS or cecal bacterial lysate secrete high concentrations of IL-12 p40.[159] These results indicate that endogenous IL-10 regulates IL-12 and IL-23 expression and that dendritic cells producing IL-12 are expanded during T_H1 driven responses. TGF-β also inhibits IL-12 responses by downregulating IL-12, IL-12RB2 and IL-12RB2 subunits.[160] Mucosal expression of IL-12 in the normal intestinal mucosa is very low, but is substantially increased in experimental T_H1-mediated colitis and human Crohn's disease. IL-12 expression is stimulated by NF-κB signaling in response to bacterial products (via TLR), TNF, IL-1β as well as IFN-γ and CD40-CD40L ligation.[161]

Signaling

IL-12 binds to a high affinity complex of 2 receptors, β1 and β2. IL-12 and IL-23 signals are mediated by JAK2 and Tyk2, which phosphorylate STAT4.[162] Accordingly, IL-12 stimulation of IFN-γ, T_H1 responses and NK cells are absent in $STAT4^{-/-}$ mice.[163]

Function

IL-12 and IL-23 polarize T_H1 responses by inducing IFN-γ and TNF. In addition, IL-12 activates NK cells and stimulates cytotoxic T cells. IL-12 p40 expression, and hence IL-12 and IL-23 activity, is markedly increased in T_H1-mediated intestinal inflammation, including experimental colitis and Crohn's disease.[72,164-167] A key role of IL-12 in the pathogenesis of intestinal inflammation is documented by potentiation of colitis by administering recombinant IL-12 or the combination of rIL-12/IL-18,[168,169] and by near complete prevention and treatment of T_H1-mediated colitis by anti-IL-12 antibody.[164,165] Fuss et al demonstrated that this pronounced protection was accompanied by apoptosis of T_H1 lymphocytes.[170] Studies of tissue IL-12 expression and antibody blockade demonstrate that IL-12 mediates the early phase, but not the late stage of colitis in IL-$10^{-/-}$ mice on a resistant C57BL/6 background.[171] A multicenter blinded study of anti-IL-12 monoclonal antibody in the treatment of Crohn's disease is in progress. Moreover, IL-12 and IL-18 can break oral tolerance in animal models, providing an additional mechanism to induce chronic inflammation.[172,173]

IL-18

This T_H1 polarizing cytokine has structural and signaling similarities to IL-1β and functional overlap with IL-12.

Structure and secretion

IL-18 is an 18 kDa single peptide chain produced as a 24 kDa precursor protein that is activated by ICE (Caspase 1) as well as proteinase 3, the same enzymes that secretion of mature IL-1β.[83,174] Like IL-1β, IL-18 lacks a leader sequence and accumulates in the cytoplasm prior to activation. Secretion of mature IL-18 is stimulated by Fas ligand.

Expression

IL-18 is produced in the intestine by epithelial cells, lamina propria macrophages and dendritic cells.[13] Expression of this cytokine is increased in active Crohn's disease, but not in ulcerative colitis.[13,175] Moreover, lamina propria mononuclear cells express IL-18 receptors.[176] LPMNC from Crohn's disease patients proliferate to a greater degree than do those from normals when stimulated with IL-18. IL-18 gene expression is upregulated by IFN-γ, IFNα/β or LPS through IFN consensus sequence-binding protein (ICSBP) and Pu.1 binding to regulatory elements on the 2 IL-18 promoters.[174]

Signaling

The IL-18 receptor is a heterodimer consisting of a ligand-binding subunit, IL-18Rα, which is identical to IL-1R-related protein, and a signaling subunit, IL-18Rβ, which is homologous to IL-1 RAcP. IL-18 binding protein (IL-18BP) is a soluble decoy receptor that can block binding of mature IL-18 with IL-18R, thereby inhibiting its biologic activity. Signaling proceeds through the IL-1R pathway involving the adapter protein MyD88, IRAK and TRAF6 (figure 2). Stimulation of this complex activates the NF-κB and MAPK/AP-1 pathways. IL-18 activation of T_H1 cells involves protein tyrosine kinase and the Src kinase LCK.

Function

IL-18 and IL-12 synergistically induce expression of IFN-γ through induction of NF-κB, AP-1 (IL-18) and STAT4 (IL-12). By itself, IL-18 has minor effects, but the combination of IL-12 and IL-18 induces far more IFN-γ in T cells than either stimulus alone. These synergistic activities are evident *in vivo*, with no intestinal inflammation induced by injection of IL-18 alone, mild disease with IL-12 alone, but severe colitis, weight loss and increased mortality with both IL-12 and IL-18.[169] This intestinal inflammation is IFN-γ dependent.[177] In addition, IL-18 stimulates production of TNF, IL-1β, C-C and C-X-C chemokines by PBMNC and production of Fas-L and TNF by T cells and NK cells.[174,178] The contribution of IL-18 to intestinal inflammation is demonstrated by consistent attenuation of experimental colitis in multiple T cell mediated and innate immune cell models (DSS, TNBS, T cell transfer to SCID mice) by a variety of IL-18 inhibitory strategies (antibody neutralization, IL-18BP binding and antisense mRNA).[98,176,179-181] Furthermore, IL-18 transgenic mice are more susceptible to low dose DSS-induced colitis, although macrophages appear to be more activated and more numerous than T lymphocytes.[182] Of interest, IL-18BP expression and IL-18/IL-18BP complexes are increased in Crohn's disease, perhaps as a compensatory protective mechanism, although free (noncomplexed) IL-18 is found at the same time.[183] As mentioned, IL-18 can break oral tolerance in mice.[173]

Other cytokines

Cytokines produced by both innate and cognate immune cells are being discovered at a rapid pace. This chapter emphasizes those molecules produced by innate immune cells that have been studied in the intestine, therefore additional molecules will be only briefly mentioned.

Chemokines

The rapidly expanding family of C-C and CXC chemokines is thoroughly covered in other comprehensive reviews[16] and is not emphasized in this discussion.

IFN-α, IFN-β

These molecules structurally related to IFN-γ are primarily secreted by activated macrophages. IFN-α has some anti-inflammatory effects, including induction of IL-1RA and sTNFRI (p55) as well as inhibition of IL-5 and IL-13. Although an uncontrolled study showed a beneficial effect of IFN-α in ulcerative colitis,[184] a controlled study showed no benefit.[185] IFN-β also has net anti-inflammatory effects, with suppression of IFN-γ and TNF expression and stimulation of IL-10 expression and T cell suppressor activity. Small open label and controlled studies with IFN-β inhibited ulcerative colitis activity.[186,187]

M-CSF and GM-CSF

These growth factors have cytokine-like properties including increasing the number and activation state of monocytes, macrophages, myeloid dendritic cells and neutrophils (GM-CSF). G-CSF and CM-CSF production by LPMNC is increased in active IBD.[112,188] A protective role for GM-CSF in Crohn's disease is supported by responses to recombinant protein in an open label study.[189] The authors suggest that the beneficial effects of GM-CSF are mediated through stimulation of bacterial killing by mucosal phagocytic cells.

Conclusions

Proinflammatory cytokines induced by activated innate immune cells have an essential role in clearing enteric pathogens, mediating acute and chronic intestinal inflammation, and stimulating the effector T cell responses. Expansion of these cytokines is dependent on central transcription factors (NF-κB, AP-1, NF IL-6, JAK/STAT) that also serve to signal responses after receptor ligation by these same gene products. This conservation of redundant signaling pathways, together with widespread expression of cytokine receptors in many mucosal cell types and recruitment of additional effector cells through chemokines, leads to amplification of the inflammatory responses. This amplification is necessary to effectively clear invading pathogens, yet can lead to irreversible tissue injury if not appropriately curtailed once the inciting stimulus is resolved. The inflammatory response is efficiently suppressed in normal hosts by redundant inhibitory molecules that are induced by proinflammatory cytokines, bacterial products and immunosuppressive molecules liberated by regulatory T cells subsets, thus restoring immunologic tolerance and homeostasis with the commensal enteric microflora. Genetically susceptible individuals who are unable to mount appropriate suppressive signals develop chronic inflammation driven by overly aggressive innate and acquired immune responses to commensal bacteria. Most of the currently available therapies effective in IBD suppress proinflammatory cytokine transcription or function by inhibiting central signaling pathways or inducing apoptosis of key effector cells. Improved understanding of the regulation of expression and mode of action of these cytokines will lead to the development of new pharmacologic inhibitors that selective block these key inducers of mucosal inflammatory responses.

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I

Attenuation of Colitis by Manipulating the Intestinal Microflora

Antibiotics with a selective aerobic or anaerobic spectrum have different therapeutic activities in various regions of the colon in interleukin-10 gene deficient mice

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Frank Hoentjen[1,2], Hermie J.M. Harmsen[3], Henri Braat[4]
Chad D. Torrice[1], Brandon A. Mann[1], R. Balfour Sartor[1]
and Levinus A. Dieleman[1]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

[3]Department of Medical Microbiology
University of Groningen, The Netherlands

[4]Department of Experimental Internal Medicine
Academic Medical Center, Amsterdam, The Netherlands

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Abstract

Background and aims

Multiple rodent models implicate resident intestinal bacteria in the pathogenesis of chronic immune-mediated intestinal inflammation. Specific pathogen-free IL-$10^{-/-}$ mice develop colitis, which does not occur in the germ-free state. We investigated whether broad or selective antibiotics affect onset and progression of disease in various regions of IL-$10^{-/-}$ mice.

Methods

Metronidazole, ciprofloxacin, vancomycin-imipenem (50 mg/kg/day) or water (control) was administered orally before (prevention) or two weeks after (treatment) colonization of germ-free IL-$10^{-/-}$ mice with specific pathogen-free bacteria. After four weeks, colonic histology scores and cytokine production by colonic explants were determined. Cecal and colonic contents were collected for quantitative bacterial analysis.

Results

In the prevention study, all antibiotics decreased inflammation in the cecum and colon. However, in the treatment study ciprofloxacin and vancomycin-imipenem decreased cecal inflammation, and reduced *E. coli* and *E. faecalis* concentrations, whereas only vancomycin-imipenem lowered direct microscopic bacterial counts. In contrast, metronidazole and vancomycin-imipenem reduced colonic injury and eliminated anaerobic bacteria including *Bacteroides* spp.

Conclusions

Both selective and broad spectrum antibiotics can prevent disease, but treatment of established colitis is more selective. Ciprofloxacin is most effective in treatment of cecal inflammation, metronidazole preferentially treats the colon, whereas vancomycin-imipenem definitively treats both regions. These results suggest that subsets of aerobic or anaerobic bacteria show regional differences in their capacity to mediate experimental colitis in IL-$10^{-/-}$ mice.

Introduction

In recent years multiple studies have emphasized the role of commensal intestinal bacteria in the pathogenesis of IBD.[1-2] In several genetically engineered and induced rodent models of chronic immune-mediated intestinal inflammation susceptible hosts develop spontaneous colitis in the presence of non-pathogenic resident intestinal bacteria, which does not occur in the germ-free state.[3]

In a well-characterized murine model of T_H1-mediated colitis IL-$10^{-/-}$ mice develop predominantly cecal inflammation shortly after weaning when susceptible strains (129 SvEv or mixed 129/C57Bl/6 background) are colonized with specific pathogen-free bacteria.[4-5] As disease progresses inflammation also develops in the ascending and transverse colon, and finally in the remainder of the colon and rectum.[4-5] In contrast, germ-free conditions prevent development of inflammation in this model, emphasizing the essential role of commensal intestinal organisms in the pathogenesis of chronic intestinal inflammation.[5] These observations are consistent with results in other models of chronic intestinal inflammation, such as in HLA-B27/β2 microglobulin transgenic rats, IL-$2^{-/-}$, TCR$\alpha^{-/-}$, SAMP-1/Yit, and CD3ε_{26} transgenic mice in which the germ-free state prevents disease development and immune activation.[6-12] The role of intestinal bacteria in the pathogenesis of experimental colitis was further emphasized after showing that broad spectrum antibiotics can both prevent and treat colitis in HLA-B27 transgenic rats and DSS-induced colitis in BALB/c mice,[13] as well as in IL-$10^{-/-}$ mice.[14]

However, it is relatively unknown how different subsets of aerobic or anaerobic bacteria influence the onset and perpetuation of experimental colitis. We describe a study in gnotobiotic IL-$10^{-/-}$ mice which were colonized with specific pathogen-free bacteria that had the following objectives: (1) investigate whether selective antibiotics affect onset and progression of colitis, (2) explore regional differences in the prevention and treatment of colonic and cecal inflammation by antibiotic administration and (3) correlate the therapeutic effects of selective antibiotic treatment with the composition of intestinal aerobic and anaerobic bacteria.

Materials and methods

Mice

Germ-free IL-$10^{-/-}$ mice (C57BL/6 x 129/Ola mixed background) were originally derived at the University of Wisconsin, Gnotobiotic Laboratory, Madison, WI, and bred at the gnotobiotic Rodent Care Facility of the Center for Gastrointestinal Biology and Disease at the College of Veterinary Medicine, North Carolina State University, Raleigh, NC.

Experimental design

IL-$10^{-/-}$ mice, 2-5 months old and raised under germ-free conditions, were transferred to specific pathogen-free conditions where bedding from *Helicobacter*-species free specific pathogen-free mice was added to each cage. The mice were colonized with *Helicobacter*-species free specific pathogen-free bacteria by oral and rectal swabbing with slurry of freshly passed feces, as described previously.[5,15]

In the prevention study metronidazole, ciprofloxacin or a combination of vancomycin-imipenem was administered in the drinking water at 50 mg/kg body weight/day starting at the time of specific pathogen-free bacterial colonization; control mice received only water. Each

group consisted of four or five mice. After four weeks the mice were killed, the cecum and various parts of the colon were collected for histology and colonic explants cultures.

In the treatment study gnotobiotic IL-10$^{-/-}$ mice did not receive any treatment for two weeks after colonization with specific pathogen-free fecal bacteria. Subsequently, metronidazole, ciprofloxacin or vancomycin-imipenem was administered in the drinking water at 50 mg/kg body weight/day; control mice received only water. Each treatment group consisted of four or five mice. At four weeks after the start of antibiotic treatment the mice were killed and the cecum and colon were collected for histology and colonic explants cultures. Cecal and colonic contents were obtained for quantitative bacteriology and FISH as described below. All studies were approved by University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Histology

At necropsy transverse and longitudinal sections of the cecum and proximal, transverse and distal parts of the colon were obtained and prepared as previously described.[5,15] Briefly, tissues were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin. A validated histologic inflammatory score ranging from 0 to 4 was used for blinded evaluation, based on the degree of cellular infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion.[5,15]

Colonic tissue fragment cultures

Cultures of colonic fragments were prepared following published methods[16] as described.[5,15] Cecal fragments were not studied because the amounts of available cecal tissue were too small. Briefly, colonic tissue was fragmented and the precise weight per well was determined, which was always in a 50-100 mg range. The colonic tissue was cultured at 37°C in 1 ml of complete RPMI medium, as described[5] and supernatants were collected after 18 hours and stored at –20°C until assay. IL-1β and IL-12 production were measured and results were normalized for the exact tissue weight used per well. Under these conditions autolysis of colonic fragments, especially epithelial cells, is expected. However, autolysis is expected to be consistent in all treatment groups, and therefore should not affect the outcome.

Cytokine measurements

We used commercially available anti-mouse IL-1β and IL-12 reagents (BD Pharmingen, San Diego, CA) in validated ELISA protocols to measure amounts of IL-1β and IL-12 in the colonic culture supernatants.[5,15] Cytokine levels were measured in duplicates and compared to standard curves using recombinant murine cytokines.

Quantitative bacteriology of cecal and colonic luminal content

Cecal and colonic contents were collected and serially diluted at 10-fold dilutions in 1x PBS. For aerobic culture the diluted contents were plated and cultured at 37°C for 24 hours on sheep blood agar (total aerobes), Mac Conkey (*E. coli*), and Bile Esculin with Azide (*Enterococci*) agar plates. For anaerobic cultures cecal and colonic contents were plated within 1 hour on BHI (total anaerobes) or *Bacteroides* Bile Esculin (BBE) agar plates in anaerobic Gas Pak jars (BBL, Cockeysville, MD) at 37°C for 48 hours. Bacterial colonies were counted, identification of specific organisms was performed by Gram staining, biochemical characterization using standard biochemical tests and confirmed by FISH, as described below. Direct microscopic bacterial counts of cecal and colonic content were assessed using a Petroffhauser counting chamber prior to bacterial culture, therefore providing an independent method of determining bacterial concentrations to complement the bacterial culture results.

Fluorescent *in-situ* hybridization

An accurately weighed portion of approximately 100 mg of cecal or colonic luminal was diluted 10 times with filtered (0.2 μm pore size) PBS. The diluted samples were homogenized by vortexing for 3 min with glass beads. The samples were diluted 1:3 with fresh 4% (w/v) paraformaldehyde in PBS, fixed overnight at 4º C and then stored at -80ºC until required. Total hybridizable cell numbers were counted by diamidino-2-phenylindole (DAPI) staining.[17] Probe BAC303 was used for the detection of the *Bacteroides/Prevotella* genera.[18] Probe EC1531 specific for *E. coli* and other *Enterobacteriaceae* was used to detect *E. coli*.[19] The probe Lab158 was used to detect *Lactobacilli* and *Enterococci.*[20] *Enterococci* were discriminated from the *Lactobacilli* using probe Efs, as described previously.[20] Dilutions of the fixed samples in PBS were applied to slides with 8 wells of 1 cm x 1 cm (CBN, Drachten, The Netherlands).[21] The slides were either hybridized with 100 μl hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS, pH 7.2) containing 5 ng/μl of one of the probes described above or stained with DAPI as described.[17] The hybridization assays were carried out overnight at 50ºC in a dark humid chamber (3 hours for the Bac303 and the EC23S probes). After hybridization, the slides were washed, air dried and subsequently mounted with Vecta Shield™ (Vector Lab., Burlingame, CA). The fluorescent cells in the samples were counted automatically with a Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany).[21] However, when the cell number was lower than $4x10^8$ cells/g wet weight the cells were counted visually using an Olympus BH2 epifluorescence microscope. All results are stated as bacterial count/g luminal content, thus normalized for the weight of luminal contents used during FISH analysis. Oligonucleotides probes were synthesized and purified by Eurogentec (Seraing, Belgium).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Data were analyzed using the paired Student's t-test or the non-parametric Mann-Whitney test. Statistical significance was defined as $P < 0.05$ compared to mice from the water control group. The Spearman correlation coefficient was computed to measure the association between histology scores and bacterial counts, denoted by r. Positive values indicate a positive correlation, negative values indicate a negative correlation, and a value of one indicates perfect correlation of the ranks. Since several comparisons were made $P < 0.01$ was considered statistically significant rather than the traditional value of 0.05.

Results

Prevention study

We first studied the ability of selective and broad spectrum antibiotics to prevent onset of colitis in germ-free IL-$10^{-/-}$ mice given antibiotics at the time of colonization with specific pathogen-free fecal bacteria.

Histology scores

Figure 1A shows that vancomycin-imipenem was most effective in preventing both cecal (0.1 ± 0.2 versus 4.0 ± 0.2 water, $P < 0.01$) and colonic inflammation (0.1 ± 0.2 versus 2.9 ± 0.2 water, $P < 0.01$, figure 1A). Ciprofloxacin and metronidazole partially prevented cecal (1.2 ± 0.3 ciprofloxacin and 2.8 ± 0.4 metronidazole versus 4.0 ± 0.2 water, $P < 0.05$ for both) and colonic

(0.7 ± 0.2 ciprofloxacin, 1.2 ± 0.2 metronidazole versus 2.9 ± 0.2 water, $P < 0.05$ for both) inflammation.

Colonic cytokine production

All antibiotics significantly decreased IL-1β production by colonic explants cultures: ciprofloxacin (2.4 ± 0.8 ng/g tissue, $P < 0.05$), metronidazole (4.0 ± 0.1 ng/g tissue, $P < 0.05$) as well as vancomycin-imipenem preventive therapy (0.4 ± 0.1 ng/g tissue, $P < 0.01$) compared to the water control group (19.6 ± 4.6 ng/g tissue, figure 1B). Colonic IL-12 production followed the same trend as IL-1β and was significantly decreased by ciprofloxacin (3.5 ± 0.8 ng/g tissue, $P < 0.05$), metronidazole (5.8 ± 0.9 ng/g tissue, $P < 0.05$), and vancomycin-imipenem treatment (1.0 ± 0.2 ng/g tissue, $P < 0.01$) compared to the water control group (14.7 ± 3.2 ng/g tissue, figure 1B).

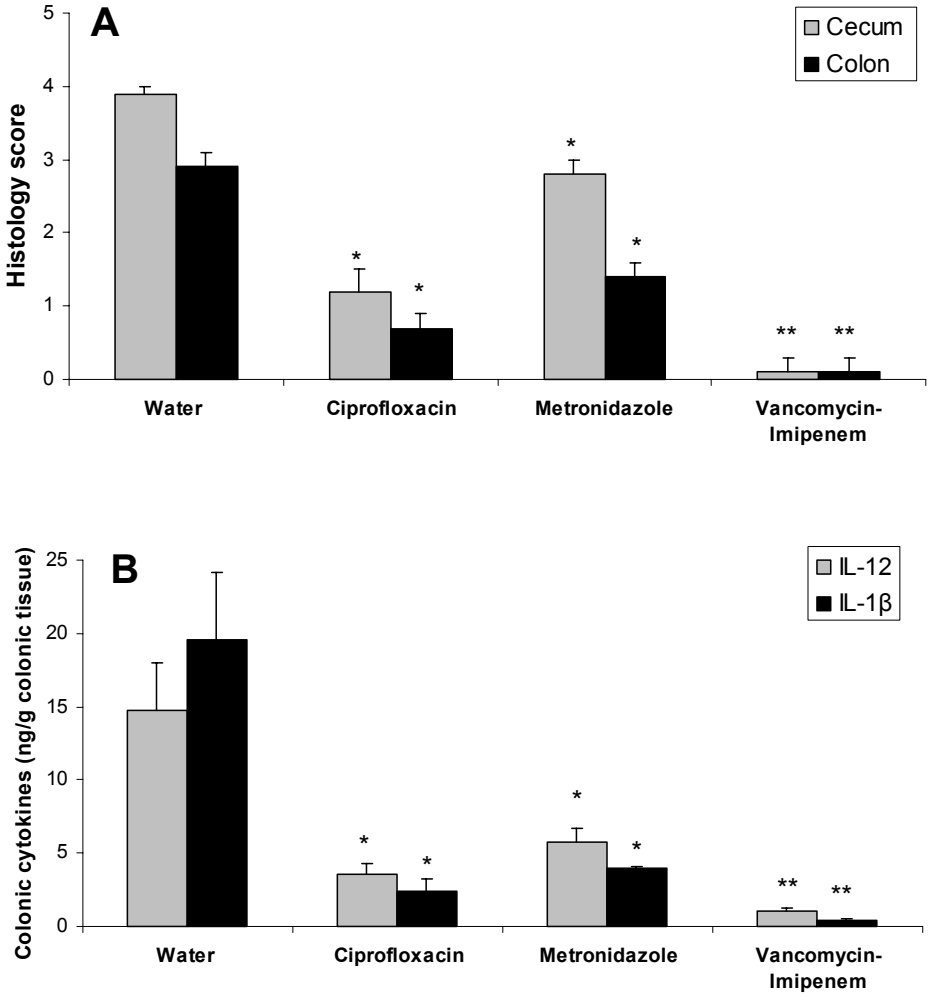

Figure 1. Prevention study. Histologic scores (0-4) and colonic cytokine secretion (ng/g colonic tissue) in gnotobiotic IL-10$^{-/-}$ mice four weeks after specific pathogen-free colonization, which were simultaneously treated with water, oral ciprofloxacin, metronidazole, or vancomycin-imipenem (50 mg/kg body weight/day). Each group consisted of four or five mice. Values represent mean ± SEM. *P < 0.05, **P < 0.01 versus untreated controls.
A: Blinded total colonic inflammatory scores.
B: Spontaneous IL-1β and IL-12 production in colonic explants cultured for 18 hours.

Treatment study

We then determined whether these antibiotics could reverse established colitis. We have previously reported that germ-free IL-10$^{-/-}$ mice colonized with specific pathogen-free bacteria

develop significant mucosal inflammation by one week of bacterial colonization, especially prominent in the cecum (cecal histologic score 2.5 ± 0.4 in specific pathogen-free versus 0.2 ± 0.2 in germ-free IL-$10^{-/-}$ mice, $P < 0.001$, colonic score 1.9 ± 0.3) which progressively increased to submucosal involvement by three weeks (histologic score cecum 2.9 ± 0.6, colon 1.8 ± 0.3).[15] Thus, germ-free IL-$10^{-/-}$ mice two weeks after colonization with specific pathogen-free bacteria have well established colitis.

Histology scores

Six weeks after specific pathogen-free colonization, and four weeks after antibiotic treatment was started, vancomycin-imipenem was most effective in reversing both established cecal (0.2 ± 0.2 versus 3.9 ± 0.2 water, $P < 0.01$) and colonic inflammation (0.2 ± 0.2 versus 3.0 ± 0.3, $P < 0.01$, figure 2A). Ciprofloxacin partially decreased cecal (1.4 ± 0.5 versus 3.9 ± 0.2 water, $P < 0.05$) but not colonic (2.3 ± 0.3, NS) inflammation, whereas metronidazole had no effect on cecal inflammation (3.4 ± 0.5, NS) but did partially treat colonic inflammation (1.0 ± 0.3 metronidazole versus 3.0 ± 0.3 water control, $P < 0.05$).

Colonic cytokine production

IL-1β production by colonic explants was significantly decreased by metronidazole (1.5 ± 0.3 ng/g tissue, $P < 0.01$) and vancomycin-imipenem (0.8 ± 0.4 ng/g tissue, $P < 0.01$) compared to the control group (18.0 ± 5.3 ng/g tissue, figure 2B). However, ciprofloxacin failed to significantly reduce colonic IL-1β production (12.2 ± 4.4 ng/g tissue, NS). IL-12 production by colonic explants was decreased by metronidazole (6.7 ± 1.2 ng/g tissue, $P < 0.05$) and vancomycin-imipenem (3.3 ± 0.3 ng/g tissue, $P < 0.05$) versus water control mice (21.4 ± 1.5 ng/g tissue, figure 2B). Ciprofloxacin did not have any effect on colonic IL-12 production (21.5 ± 1.3 ng/g tissue, NS) compared to the water control group.

Quantitative bacteriology of cecal and colonic contents

Direct microscopic bacterial counts of cecal contents were 1-2 log lower in the vancomycin-imipenem group compared to water controls and was unchanged from controls in ciprofloxacin or metronidazole treated mice (table 1). Although DAPI-stained cell counting only showed a 5-fold reduction of total microbial cells in vancomycin-imipenem-treated mice versus other groups, most of these were identified as yeasts (table 2). The number of cecal total aerobic bacteria was significantly lower in ciprofloxacin-treated mice versus other groups. The amounts of *E. coli* and *Enterococci* spp were significantly decreased by ciprofloxacin and vancomycin-imipenem treatment (table 1).

Metronidazole treatment significantly increased amounts of luminal *E. coli*. These results were confirmed by FISH of the same cecal contents, which also identified that *Enterococci* spp were mainly *E. faecalis* (table 2). Total anaerobic bacterial amounts were significantly decreased by metronidazole as well as vancomycin-imipenem treatment with far greater effects seen with the combination treatment, as shown in table 1. FISH detected large populations of *Lactobacilli* in the control mice, which were significantly decreased by ciprofloxacin and eliminated by vancomycin-imipenem treatment. Both metronidazole and vancomycin-imipenem decreased *Bacteroides* spp amounts below detectable levels, which was confirmed by FISH (table 1 and 2). No significant differences were observed between quantitative bacteriological results from cecal versus colonic bacterial contents (data not shown).

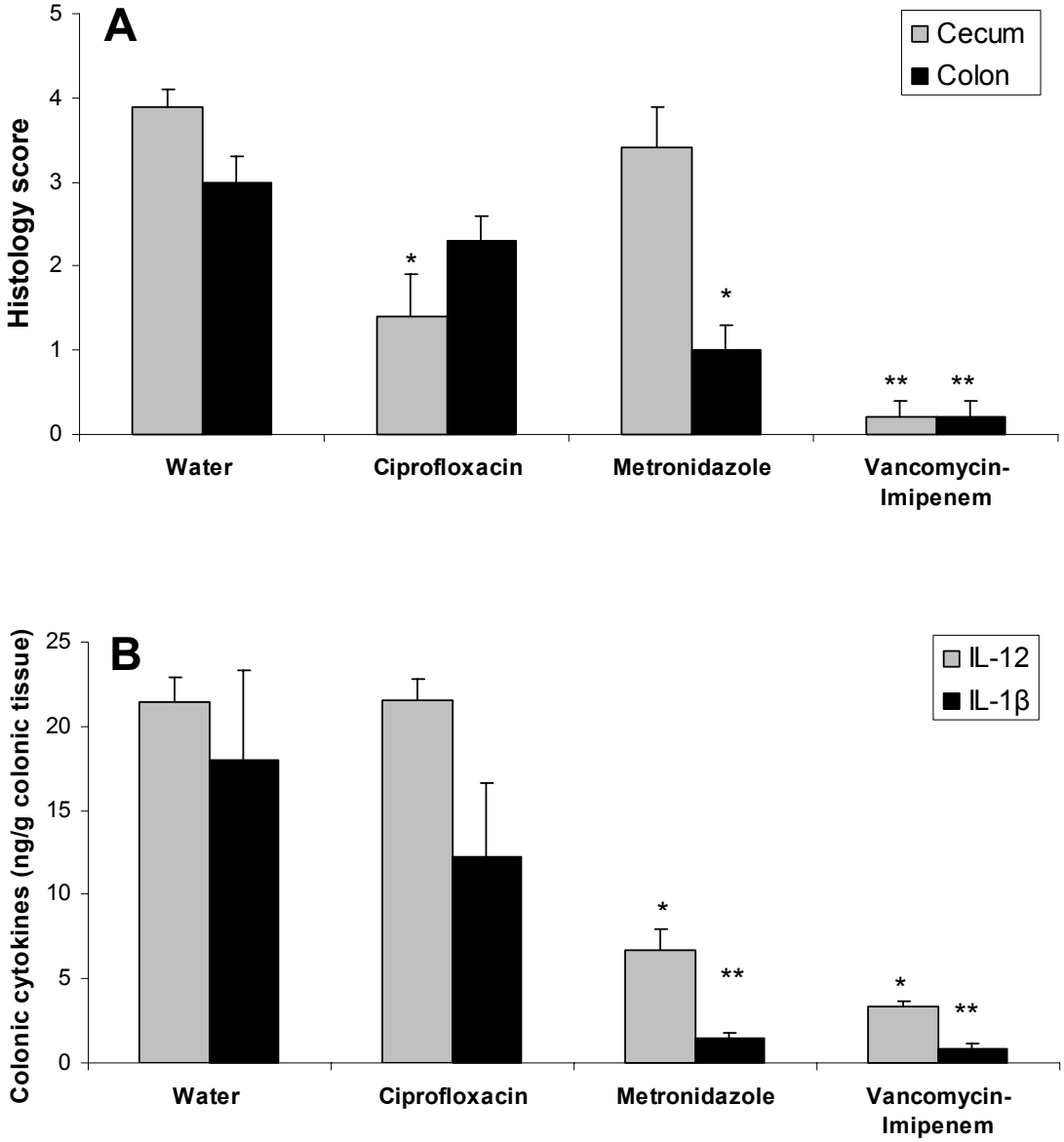

Figure 2. Treatment study. Histologic scores (0-4) and colonic cytokine secretion (ng/g colonic tissue) in gnotobiotic IL-10$^{-/-}$ mice six weeks after specific pathogen-free colonization. Two weeks after specific pathogen-free colonization resulting in established colitis, antibiotic treatment (50 mg/kg body weight/day) started with water, oral ciprofloxacin, metronidazole, or vancomycin-imipenem. Each group consisted of four or five mice. Values represent mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus untreated controls.
A: Blinded total colonic inflammatory scores.
B: Spontaneous IL-1β and IL-12 production in colonic explants cultured for 18 hours.

We then investigated statistical correlations between histologic inflammation and bacterial counts, using the Spearman correlation coefficient as a nonparametric measure of the correlation of ranks. The correlation of ranks instead of the correlation of the raw data was computed because the data were skewed and the assumptions required to do hypothesis testing on the parametric correlation coefficient were not satisfied. We merged all treatment groups, to see whether we could show a threshold for either total microbes or any of the species cultured, related to the perpetuation of colitis.

Treatment	Microscopic Bacterial Counts ($x10^8$)	Total Aerobes ($x10^7$)	*E. coli* ($x10^7$)	*Enterococci* ($x10^7$)	Total Anaerobes ($x10^8$)	*Bacteroides* spp. ($x10^7$)
Water	38 ± 12	5 ± 2	7 ± 3	48 ± 20	86 ± 30	32 ± 8
MNZ	43 ± 7	20 ± 10	84 ± 13[a]	57 ± 30	4.8 ± 5[b]	< 0.001[a]
Cipro	24 ± 6	< 0.001[a]	< 0.001[a]	0.2 ± 0.1[a]	96 ± 50	28 ± 5
V + I	1 ± 0.1[a]	21 ± 10	< 0.001[a]	< 0.001[a]	< 0.001[a]	< 0.001[a]

Table 1. Quantitative bacteriology of cecal content by bacterial cultures. Direct microscopic bacterial counts and cecal concentrations of aerobic bacteria, *E coli*, *Enterococci*, as well as total anaerobic bacteria, including *Bacteroides* spp, in gnotobiotic IL-$10^{-/-}$ mice colonized with specific pathogen-free flora and treated with ciprofloxacin (Cipro), metronidazole (MNZ) or vancomycin-imipenem (V+I). Values are given as mean ± SEM colony forming units per gram cecal contents. [a] P< 0.01 versus water controls, [b] P< 0.05 versus water controls.

Direct microscopic bacterial counts strongly correlated with cecal inflammation (r = 0.77, *P* = 0.0002), but to a lesser extent with colonic inflammation (r = 0.53, *P* = 0.02). Total aerobic counts did not correlate with overall disease, and in fact were negatively correlated with colonic inflammation (r = -0.73, *P* = 0.0006). Cecal but not colonic inflammation correlated with *E. coli* concentrations (r = 0.76, *P* = 0.0003) and with *E. faecalis* concentrations (r = 0.70, *P* = 0.001). In contrast, colonic but not cecal inflammation strongly correlated with total anaerobes (r = 0.79, *P* = 0.0001) and *Bacteroides* spp. (r = 0.89, *P* = 0.0001).

Treatment	Total Bacterial Count (x 10^8)	*E. coli* (x 10^7)	*E. faecalis* (x 10^7)	*Bacteroides* spp (x 10^7)	*Lactobacilli* spp (x 10^7)
Water	86 ± 21	6 ± 2	40 ± 38	114 ± 39	141 ± 40
MNZ	85 ± 9	171 ± 47[a]	78 ± 40	<1[a]	247 ± 48
Cipro	31 ± 7	<1[a]	<1[a]	61 ± 12	11 ± 5[a]
V + I	18 ± 1[a]	<1[a]	<1[a]	<1[a]	<1[a]

Table 2. Quantitative bacteriology of cecal content by FISH. Cecal luminal concentrations of *E coli*, *Enterococci*, *Bacteroides* spp, and *Lactobacilli* spp in gnotobiotic IL-$10^{-/-}$ mice colonized with specific pathogen-free flora and treated with ciprofloxacin (Cipro), metronidazole (MNZ) or vancomycin-imipenem (V+I). Values are given as mean ± SEM per gram of cecal content, measured by fluorescent *in-situ* hybridization using probes specific for 16S ribosomal RNA of *E. coli*, *E. faecalis*, *Bacteroides spp*, and *Lactobacilli* spp. <1 below detection limit of 10^7 bacteria/gram content. [a] *P*< 0.01 versus water controls.

Discussion

Bacteria and their products play a crucial role in the pathogenesis of chronic intestinal inflammation in animal models and human IBD, providing therapeutic potential for antibiotics to manipulate the intestinal flora in order to influence the onset and course of disease.[1-2] Germ-free IL-10$^{-/-}$ mice do not have colitis in the absence of intestinal bacteria, but we showed previously that they rapidly develop a T_H1-mediated transmural acute and chronic inflammation within 1 week after colonization with specific pathogen-free bacteria.[15] Therefore, following identical protocols,[15] this model provided us the opportunity to investigate established colitis two weeks after specific pathogen-free colonization. In our studies 4-6 weeks after specific pathogen-free bacteria, the cecum was consistently more affected than the colon in IL-10$^{-/-}$ mice.

We first showed that the broad spectrum combination vancomycin-imipenem almost completely prevented and treated both cecal and colonic inflammation, in agreement with previous reports by several authors in various models. A combination of metronidazole and neomycin prevented and treated colitis in IL-10$^{-/-}$ mice.[14] Vancomycin-imipenem prevented and treated colitis in HLA-B27 rats, DSS-induced colitis in mice,[13] as well as TNBS-induced experimental colitis in rats.[22] However, although the combination of ciprofloxacin and metronidazole prevented and treated inflammation in the SAMP1/Yit spontaneous ileitis model[23] it improved acute but not chronic DSS-induced colitis in mice.[24] In human studies the combination of ciprofloxacin and metronidazole was reported to be effective in treating refractory pouchitis[25] as well as active Crohn's disease.[26-27]

We also showed that selective antibiotics such as ciprofloxacin or metronidazole could prevent cecal and colonic inflammation in gnotobiotic IL-10$^{-/-}$ mice after specific pathogen-free colonization, but only selectively reversed established colitis. The higher efficacy of selective antibiotics for prevention rather than treatment of colitis is in agreement with previous studies. For example, ciprofloxacin prevented the induction of colitis in IL-10$^{-/-}$ mice born under specific pathogen-free conditions, but showed only minor effects in established colitis.[14] Metronidazole prevented DSS-induced colitis in mice, but this antibiotic had no effect after the onset of colitis.[24] In HLA-B27 transgenic rats and DSS-treated mice, oral administration of either ciprofloxacin or metronidazole could prevent colitis but was less effective in treating established inflammation.[13] The greater efficacy of selective antibiotics for preventing colitis may be explained by different roles for various endogenous bacterial species in the different phases of the inflammatory process. As proposed by Rath et al, some bacterial species might initiate inflammation while others, perhaps including a larger spectrum of intestinal bacteria, perpetuate disease.[13] Therefore, it is easier to prevent onset of colonic inflammation than to treat established disease. These findings are in agreement with the ability of recombinant IL-10 to prevent but not treat experimental chronic granulomatous inflammation induced by bacterial cell wall polymers in rats.[28] This might also explain the relative lack of efficacy by metronidazole and ciprofloxacin in the treatment of ileal Crohn's disease,[29] whereas metronidazole or ornidazole could prevent recurrence of Crohn's disease in the neoterminal ileum after ileal resection and primary ileocolonic anastomosis.[30-31]

A unique feature of our study is the observation that selective antibiotics showed regional differences in treating established colitis in IL-10$^{-/-}$ mice. Ciprofloxacin was most beneficial in the cecum, but did not treat colonic inflammation, whereas metronidazole was more effective in the colon. On the other hand, broad spectrum antibiotics vancomycin-imipenem were superior to ciprofloxacin or metronidazole alone and successfully treated both intestinal regions. Regional specificity of antibiotics is seen in human Crohn's disease in which metronidazole, either alone or in combination with ciprofloxacin, is more effective in colonic versus ileal Crohn's disease,[27,29,32]

although in a small study ciprofloxacin did treat Crohn's ileitis.[33] In two other studies efficacy of ciprofloxacin in relation to disease localization was not mentioned.[34-35] In our study cytokine production by colonic explants, normalized for tissue weight, was significantly reduced by metronidazole but not by ciprofloxacin, demonstrating the specificity of ciprofloxacin for the cecal region rather than the colon.

Treatment with vancomycin-imipenem correlated with a 1-2 log decrease of total luminal bacteria, while other treatments did not significantly decrease total luminal bacterial concentrations. Similar effects on total cecal bacterial load by this antibiotic combination have been reported in HLA-B27 transgenic rats[13] as well as by metronidazole plus ciprofloxacin treatment in the SAMP/Yit mouse model,[23] most likely due to emergence of resistant bacterial species. Our results confirm the importance of luminal antigenic bacterial load in the perpetuation of chronic colitis, since the broad spectrum antibiotic combination almost totally eliminated disease. When all treatment groups were merged, there was a significant correlation between direct microscopic bacterial counts and histology scores in both the cecum and colon. However, reduction of bacterial load is not the only mechanism involved in response to treatment, because 1) selective antibiotics have selective therapeutic effects in different large intestinal regions, despite the lack of effect on direct microscopic bacterial counts in IL-10$^{-/-}$ mice, 2) vancomycin-imipenem was more effective than ciprofloxacin in reducing cecal inflammation, but did not decrease the total aerobic count, 3) decreased luminal *E. coli* and *E. faecalis* concentrations, but not the total aerobic counts, correlated with reduced cecal inflammation. Therefore, our results suggest that not only reduced bacterial load but also specific subsets of aerobic bacteria like *E. coli*, might preferentially perpetuate cecal inflammation, while anaerobic bacteria, including *Bacteroides*, cause colitis. This hypothesis is consistent with the selective effects of ciprofloxacin in the cecum and metronidazole in the colon. Ciprofloxacin is most effective against enteric aerobic Gram-negative organisms, and in our study it significantly reduced total cecal and colonic aerobic bacteria, with undetectable *E. coli* and a significantly reduced amount of luminal *Enterococci* spp. Interestingly, in preliminary studies, IL-10$^{-/-}$ mice monoassociated with *E. coli* preferentially develop cecal inflammation.[36] *E. coli* could therefore play a role in the (induction and) perpetuation of cecal inflammation in specific pathogen-free IL-10$^{-/-}$ mice. Despite elevated amounts of cecal *E. coli* in HLA-B27 transgenic rats with severe chronic colitis,[37] monoassociation of these rats with *E. coli* did not induce colitis in these rats,[38] indicating that the ability of *E. coli* to induce and perpetuate chronic colitis is host-dependent. The potential role of *E. coli* in chronic intestinal inflammation was further emphasized by the observation that ileal mucosa from Crohn's patients with postoperative recurrence contained more enteroadherent/invasive *E. coli* strains than healthy controls.[39] In addition, IBD patients have increased serum and mucosal antibody responses to *E. coli.*[40-41] Monoassociation of IL-10$^{-/-}$ mice with *E. faecalis* induces a distal colitis with slow onset of disease, starting at 12 weeks of colonization.[36,42-43] Although ciprofloxacin reduced cecal inflammation and luminal concentrations of *E. faecalis* in our study, the regional localization (distal colon versus cecum) and the slow onset of disease in monoassociated IL-10$^{-/-}$ mice make it unlikely that *E. faecalis* plays an important role in cecal inflammation. Additionally, it also appears unlikely that *E. faecalis* perpetuates established distal colitis in IL-10$^{-/-}$ mice colonized with specific pathogen-free bacteria for four to six weeks, since metronidazole, which selectively decreased colonic inflammation, had no effect on *E. faecalis* concentrations.

Metronidazole is selectively active against anaerobic bacteria, including colonic Gram-negative *Bacteroides* spp.[44] Metronidazole effectively treated colonic inflammation in our IL-10$^{-/-}$ mice, significantly reduced luminal anaerobic bacteria and eliminated *Bacteroides* spp, in both the

cecum and colon, suggesting that differences in local luminal concentrations of these organisms could not explain the regional variation of colonic inflammation. Thus, anaerobic bacteria appear to have little effect in cecal inflammation in this model, but could play an important role in colonic inflammation. In selective colonization studies, a cocktail of six enteric bacteria, including *B. vulgatus*, induced minimal colitis in gnotobiotic IL-$10^{-/-}$ mice,[5] although monoassociation with *B. vulgatus* induced colitis in carrageenan-fed guinea pigs and HLA-B27 transgenic rats.[38,45] Additionally, Mann et al demonstrated that different *Bacteroides* spp show variability in pathogenic capacity, with *B. vulgatus* and *Bacteroides thetaiotamicron* but not *Bacteroides distasonis* inducing colitis in monoassociated HLA B27 transgenic rats.[46] In our study, host-specificity and pathogenic variability of anaerobic species might determine which organisms mediate colonic inflammation. Thus, it appears likely that metronidazole eliminates other anaerobic intestinal organisms than *B. vulgatus*, which could mediate established colitis but not cecal inflammation in IL-$10^{-/-}$ mice.

Administration of several *Lactobacillus* spp can both prevent and treat colitis in IL-$10^{-/-}$ mice.[47-48] However, our FISH analysis demonstrated that both vancomycin-imipenem and ciprofloxacin treatment eliminated *Lactobacilli* spp, whereas metronidazole did not affect endogenous luminal *Lactobacilli* spp. Therefore, the beneficial effects of antibiotics are not mediated by increased *Lactobacilli* spp levels.

In conclusion, selective antibiotics and broad spectrum combination antibiotics can prevent development of inflammation in both the cecum and colon of IL-$10^{-/-}$ mice after colonization with specific pathogen-free bacteria. In contrast, treatment of established colitis is more selective in its regional therapeutic response. Ciprofloxacin is most effective in cecal inflammation and reduces aerobic organisms, including *E. coli* and *E. faecalis*, whereas metronidazole is preferentially active in the colon and selectively decreases anaerobic bacteria and *Bacteroides* spp. Vancomycin-imipenem treats both regions very effectively, reduces total luminal bacteria and eliminates specific aerobic and anaerobic organisms. These results suggest that specific subsets of aerobic and anaerobic intestinal bacteria selectively perpetuate colitis in the cecum and the colon in this experimental model of chronic intestinal inflammation.

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Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment

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Levinus A. Dieleman[1], Marije Goerres[1], Annemarie Arends[1]
Dave Sprengers[1], Chad D. Torrice[1], Frank Hoentjen[1,2]
Tony B. Grenther[1], and R. Balfour Sartor[1]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

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Abstract

Background and aims

B. vulgatus induces colitis in gnotobiotic HLA-B27 transgenic rats, while broad spectrum antibiotics prevent and treat colitis in specific pathogen-free transgenic rats, although disease recurs after treatment ends. *Lactobacilli* treat human pouchitis and experimental colitis. We investigated if *Lactobacillus GG* can prevent colitis in transgenic rats monoassociated with *B. vulgatus* and if *Lactobacillus GG* or *Lactobacillus plantarum* can treat established colitis in specific pathogen-free transgenic rats and prevent recurrent disease after antibiotics were stopped.

Methods

1. Germ-free HLA-B27 transgenic rats were monoassociated with *B. vulgatus* for four weeks following two weeks colonization with *Lactobacillus GG* or no bacteria. 2. Specific pathogen-free B27 transgenic rats received oral vancomycin-imipenem for two weeks, or water alone, followed by four weeks treatment with either oral *Lactobacillus GG, Lactobacillus plantarum* or water only. Disease activity was quantified by blinded gross and histologic scores, cecal MPO, IL-1β, TNF, TGF-β and IL-10.

Results

Lactobacillus GG did not prevent colitis in *B. vulgatus*-coassociated transgenic rats nor treated established disease in specific pathogen-free rats. However, *Lactobacillus GG* but not *Lactobacillus plantarum* prevented colitis relapse in antibiotic-treated rats with reduced gross and histologic scores, cecal MPO, IL-1β and TNF, whereas cecal IL-10 was increased.

Conclusions

Lactobacillus GG did not prevent colitis in gnotobiotic transgenic rats nor treated established disease in specific pathogen-free rats, but was superior to *Lactobacillus plantarum* in prevention of recurrent colitis. These studies suggest that antibiotics and probiotic agents provide synergistic therapeutic effects, perhaps mediated by altered immunomodulation with selective activity of different *Lactobacillus* species.

Introduction

The role of intestinal bacteria in the pathogenesis of human IBD, particularly Crohn's disease, is well-recognized.[1-2] These diseases typically occur at sites with the highest concentrations of intestinal bacteria, such as the colon and terminal ileum. Antibiotics and fecal diversion are effective treatment modalities for Crohn's disease,[1-3] whereas re-establishing continuity of the bypassed distal bowel or infusion of intestinal contents into the excluded ileum leads to disease recurrence.[4]

The role of intestinal bacteria in the initiation and perpetuation of chronic intestinal inflammation is most convincingly demonstrated in experimental models of chronic intestinal inflammation in which genetically susceptible hosts develop spontaneous colitis in the presence of commensal intestinal organisms, whereas no disease occurs in the germ-free state.[5, 6-8]

HLA-B27 transgenic rats develop colitis, gastritis, and systemic inflammation in an specific pathogen-free environment.[9] However, germ-free transgenic rats and non-transgenic rats colonized with specific pathogen-free bacteria show no evidence of colitis, gastritis, and arthritis.[5,10] Colitis and gastroduodenal inflammation in transgenic rats is attenuated by metronidazole therapy and can be prevented and treated with broad spectrum antibiotics.[11] Moreover, cecal bacterial overgrowth within an experimental blind loop exacerbates colitis, whereas a bypass of the cecum attenuates disease in this model, which correlates with the concentrations of luminal *Bacteroides* species.[12] *B. vulgatus* preferentially induces colitis in transgenic rats after monoassociation for 4 weeks, whereas monoassociation with *E. coli* does not cause disease.[13] These findings indicate that not all bacteria are equal in their capacity to induce colitis.

Probiotics are defined as living commensal micro-organisms that are important to the health and well-being of the host.[14] *Lactobacilli*, although not predominant enteric organisms, are present throughout the GI tract of healthy humans and rodents. Several *Lactobacillu*s strains, such as *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus fermentum and Lactobacillus GG* are able to colonize the human GI tract.[15] Several human clinical trials have shown efficacy of several species of probiotics in viral-, and bacterial-induced infectious colitis as well as in antibiotic-associated diarrhea.[14,16-17] Rectal administration of native murine *Lactobacillus reuteri* prevented colitis in IL-10$^{-/-}$ mice and a similar effect was shown by the oral prebiotic lactulose, which stimulated the growth of endogenous *Lactobacilli* spp.[18] Continuous oral treatment of *Lactobacillus plantarum* in germ-free IL-10$^{-/-}$ mice colonized with specific pathogen-free flora could attenuate established colitis.[19]. A probiotic preparation including four species of *Lactobacilli*, three *Bifidobacteria* and one *Streptococcus salivarius* (VSL# 3) maintained remission of refractory pouchitis after transient antibiotic therapy.[20] This probiotic cocktail VSL#3 was also beneficial in the treatment of colitis in IL-10$^{-/-}$ mice.[21]

The first aim of our study was to investigate if the probiotic species *Lactobacillus GG* can prevent colitis in HLA-B27 transgenic rats monoassociated with the disease-inducing *B. vulgatus*. Secondly, we determined if *Lactobacillus GG* or *Lactobacillus plantarum* could treat established colitis in specific pathogen-free HLA-B27 transgenic rats or prevent recurrent disease after broad spectrum antibiotics were stopped. Thirdly, we studied the protective mechanisms of probiotics in the prevention of relapse model.

Materials and methods

Rats

Transgenic rats of the 33-3 line on an inbred F344 background, bearing several copies of the genes for HLA-B27 and human β2 microglobulin, [9] and their non-transgenic littermates were originally obtained from Dr. Joel Taurog, Southwestern Medical School, Dallas, TX. After Caesarian section they were housed in Trexler isolators in germ-free conditions at the Gnotobiotic Core facility at the College of Veterinary Medicine at Raleigh, NC. Specific pathogen-free transgenic rats and their non-transgenic littermates were born and maintained in filter-top cages in a specific pathogen-free environment.

Bacteria

Freeze-dried cultures of *Lactobacillus GG*, which was *Lactobacillus rhamnosus* strain GG, and *Lactobacillus plantarum* strain 299v were kindly provided by ConAgra, Omaha, NE. Before administration Lactobacilli strains were grown aerobically overnight at 37°C in MRS broth. The next day the bacteria were harvested by centrifugation. After washes bacteria were resuspended in sterile PBS and added to the drinking water. Quantification and purity of bacterial suspensions as bacterial CFU was performed in serial dilutions of the drinking water on *Lactobacillus* MRS-Vancomycin plates. *B. vulgatus* was kindly provided by Dr. A.B. Onderdonk, (Harvard University, Cambridge, MA) and was originally derived from guinea pigs with carrageenan-induced colitis.[22] This strain has been used to induce colitis in monoassociated transgenic rats.[13] *B. vulgatus* was cultured anaerobically in BHI broth before colonization of gnotobiotic rats.

Experimental design

Prevention study

In the first part of the study germ-free transgenic rats and their non-transgenic littermates were divided into three groups of six rats each at the age of two months. The first two groups were monoassociated with either *B. vulgatus* or *Lactobacillus GG* for four weeks, the third group was selectively colonized with *B. vulgatus* for four weeks, but was colonized with *Lactobacillus GG* for two weeks before co-colonization with *B. vulgatus*. Colonization with *B. vulgatus* was performed by oral and rectal swabbing using stool from other *B vulgatus*-monoassociated rats. *Lactobacillus GG* or *Lactobacillus plantarum* was administered once in the drinking water at 10^{10} CFU per rat per day after overnight culture at 37°C in MRS broth. The amount of bacteria consumed by each rat per day was calculated by measured consumption of 30 ml of water per day times $3.3x10^8$ CFU/ml = 10^{10} CFU. Successful colonization was assessed after one week by plating stool suspensions on BBE and MRS plates for *Bacteroides* spp and *Lactobacillus* respectively for a two days anaerobic culture, as well as by performing Gram stains. No contaminating organisms were discovered. Rats were killed after four weeks of *B. vulgatus* or *Lactobacillus GG* colonization. At necropsy ceca and colons were fixed in 10% buffered formalin for histological evaluations. Ceca were also snap-frozen for subsequent MPO and cytokine analysis. The cecal contents were collected for microbiological analysis as described below.

Prevention of relapse study

At 10 weeks of age specific pathogen-free HLA-B27 transgenic rats were divided into the following four treatment groups, each consisting of six rats; group one received only drinking water ad libitum for six weeks, group two received 50 mg/kg each of vancomycin-imipenem in

their drinking water for two weeks, followed by four weeks of water, group three received 50 mg/kg each of vancomycin-imipenem for two weeks, followed by daily administration of *Lactobacillus GG* or *Lactobacillus plantarum* in the drinking water at 10^{10} CFU/rat/day during four weeks, group four received two weeks of water, followed by four weeks of 10^{10} CFU/rat/day *Lactobacillus GG* or *Lactobacillus plantarum* in their drinking water. All rats were killed six weeks after the start of treatment. Ceca and colons were collected for histology and snap frozen for MPO and cytokine analysis as described below. Cecal contents were collected for quantitative microbiological analysis.

Histology

Tissues were prepared as previously described.[10] A validated histological inflammatory scoring ranging from 0-4 was used for blinded evaluation of colonic and cecal inflammation.[10]

Cecal cytokine analysis

Frozen cecal tissues were thawed and lysed in PBS containing a cocktail of protease inhibitors (antipain, aprotinin, leupeptin, pepstatin A, all at 1 μg/ml and 1 mM PMSF (Sigma, St Louis, MO).[23] Tissue was then homogenized, after which the homogenate was assayed for IL-1β, TNF, IL-10 and TGF-β. Concentrations of interleukin-1β were measured by a rat IL-1β ELISA developed by S. Poole, as described.[10] Cecal TNF and IL-10 concentrations were determined by rat cytokine-specific ELISAs. TGF-β1 concentrations were measured after acidification and neutralization according to the manufacturer using a TGF-β1- specific ELISA (Promega, Madison,WI).

MPO assay

Homogenized ceca were assayed for MPO activity (units per gram of tissue) as described previously.[24]

Determination of cecal bacterial concentrations

Cecal contents were removed aseptically, immediately placed into an anaerobic chamber and dissolved in sterile pre-reduced PBS. Serial 100-fold dilutions were plated and cultured anaerobically for two days on *Lactobacilli* spp MRS-vancomycin agar for quantification of *Lactobacillus*, on BBE agar plates for *Bacteroides* spp, and on Brucella agar (Remel, Lenexa, KS) for total anaerobic bacteria. Morphology was determined by Gram staining. Results were expressed as CFU per gram cecal contents dry weight.

Inhibition of in vitro adherence to IEC-6 cells

The rat epithelial cell line IEC-6 cells (ATCC # CRL 1592, Rockville, MD) were grown to confluence in a 12-well tissue culture plate in antibiotic-free DMEM containing 5% heat-inactivated newborn bovine serum and 10 IU (International Units)/ml insulin (Life Technologies, Burlington, ON, Canada).

Intestinal epithelial adherence assay was performed according to Mack et al.[25] Briefly, after removal of non-adherent cells, an adherent IEC-6 monolayer was incubated at 37°C with 10^9 CFU *B. vulgatus* in the presence or absence of 10^9, 10^8 or 10^7 CFU *Lactobacillus GG* per well in 2 ml of antibiotic-free complete medium under aerobic conditions. After 3 hours incubation non-adherent bacteria were removed by washing three times with warm PBS, IEC-6 cells plus adherent bacteria were collected by trypsinization. Collected cells and adherent bacteria were then

serially diluted and plated anaerobically on BHI plates for 48 hours. Bacteria were counted by colony identification, confirmed by Gram staining.

Statistical analysis

Statistics were performed using the Student t test or Mann-Whitney test for comparison between the groups. Significance was considered to be $P < 0.05$. All data are presented as mean ± SEM.

Results

Prevention study

Monoassociation of transgenic rats with *B. vulgatus* resulted in mild cecal and colonic inflammation (histology score cecum 1.5 ± 0.1 and colon 1.2 ± 0.1). However, pretreatment of germ-free transgenic with *Lactobacillus GG* followed after two weeks by co-association with *B. vulgatus* did not prevent the development of mild colitis induced by *B. vulgatus* (histology scores cecum 1.6 ± 0.2 and colon 1.1 ± 0.2). However, monoassociation of gnotobiotic transgenic rats with *Lactobacillus GG* did not induce colitis (cecal and colonic scores both 0.2 ± 0.1). Similarly, gnotobiotic non-transgenic rats colonized with *B. vulgatus*, *Lactobacillus GG* or both did not develop colitis (histology score cecum or colon 0.2 ± 0.1).

Cecal IL-1β concentrations from *B. vulgatus*-monoassociated transgenic rats (12 ± 3 pg/mg cecal wet weight) were not significantly different from those obtained from *B. vulgatus*- and *Lactobacillus GG* -co-associated transgenic rats (11 ± 2 pg/mg). Cecal IL-1β concentrations from *Lactobacillus GG*-monoassociated transgenic rats (5 ± 2 ng/g) were similar to those from gnotobiotic non-transgenic littermates colonized with *B. vulgatus*, *Lactobacillus GG* or both (2 ± 1 pg/mg).

The concentration of *Bacteroides* spp in the cecal content of transgenic rats co-associated with *B. vulgatus* and *Lactobacillus GG* did not significantly differ from *B. vulgatus*-monoassociated transgenic rats ($61 \pm 8 \times 10^9$ versus $47 \pm 4 \times 10^9$ CFU/g cecal content in *B. vulgatus* and *B. vulgatus* plus *Lactobacillus GG*-associated transgenic rats respectively). The concentration of cecal *Lactobacillus GG* ($0.45 \pm 0.04 \times 10^9$ CFU/g) in the *B. vulgatus* plus *Lactobacillus GG*-co-associated rats was 100 fold lower than the *B. vulgatus* concentrations, but did not significantly differ from the concentration of cecal *Lactobacillus GG* in *Lactobacillus GG*-monoassociated transgenic rats ($0.49 \pm 0.10 \times 10^9$ CFU/g). Gram staining, aerobic and anaerobic culture of cecal contents from the gnotobiotic rats excluded contamination with other organisms.

Prevention of colitis relapse in specific pathogen-free HLA-B27 transgenic rats

Oral administration of broad spectrum antibiotics (oral imipenem-vancomycin) for two weeks to specific pathogen-free HLA-B27 transgenic rats followed by cessation of this treatment resulted in relapse of colitis after four weeks. The cecal gross gut scores (1.7 ± 0.2) as well as total colonic histology scores (1.7 ± 0.2) of antibiotic-water treated rats four weeks after stopping antibiotics did not differ from untreated control transgenic animals (cecal gross score 1.7 ± 0.2, total colonic histology score 1.7 ± 0.1) (figures 1-3). Also *Lactobacillus GG* administration alone could not effectively treat disease (cecal gross score 1.3 ± 0.3, total colonic histology score 1.8 ± 0.2). However, if the antibiotic treatment was followed by daily oral administration of *Lactobacillus GG*, relapse of colitis could be partially prevented, as shown by a significant decrease of cecal gross score (0.6 ± 0.1) and total colonic histologic inflammatory score (1.1 ±

0.3) of this treatment group (figures 1-3). In contrast to *Lactobacillus GG* treatment, oral administration of *Lactobacillus plantarum* alone or after antibiotic treatment had no effect; neither cecal gross scores (data not shown) nor colonic histology scores (figure 3) differed significantly between treatment groups.

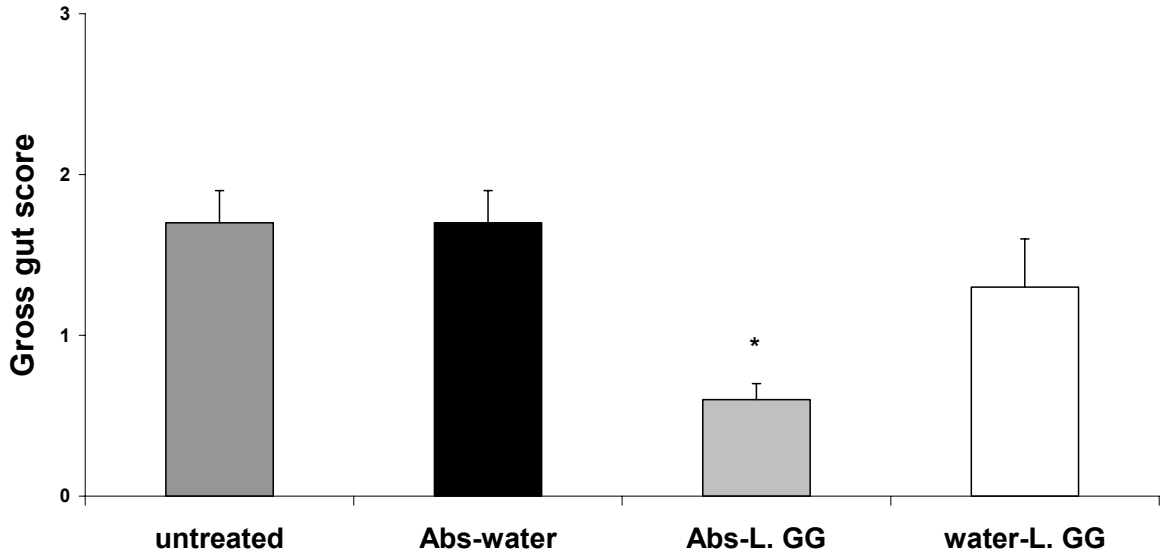

Figure 1. Gross gut scores (GGS) (0-4) of ceca from specific pathogen-free transgenic rats treated with vancomycin-imipenem (Abs) or water followed by oral *Lactobacillus GG* or water administration. GGS from untreated specific pathogen-free transgenic rats are also given. Each group consisted of six rats. Data are expressed as the mean ± SEM. * $p < 0.01$ versus untreated control rats.

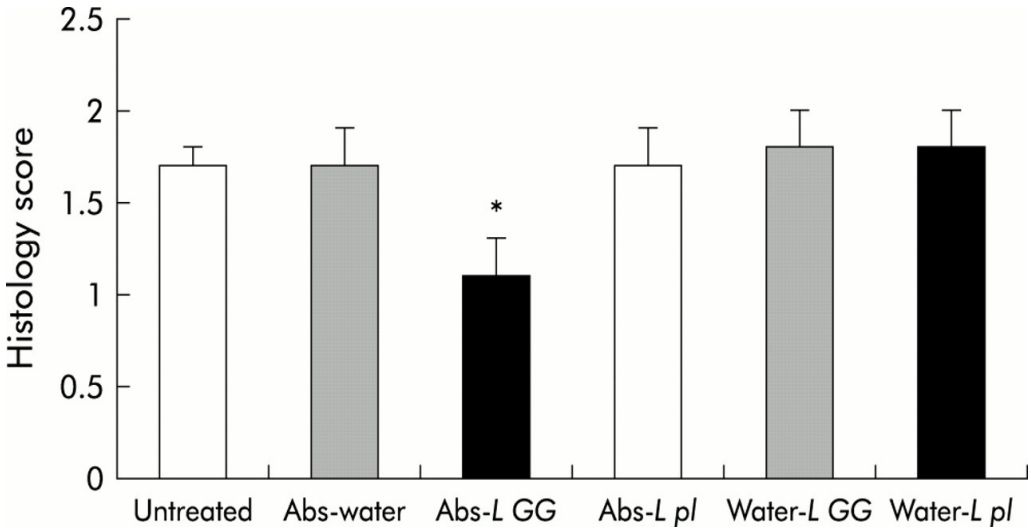

Figure 2. Blinded total colonic inflammatory scores in specific pathogen-free transgenic rats treated with two weeks oral broad spectrum antibiotics (Abs) or water followed by four weeks *Lactobacillus GG*, *Lactobacillus plantarum (L. pl)* or water. Also histology scores from specific pathogen-free transgenic untreated rats were given. Values represent mean ± SEM of total histology scores (0-4). Vancomycin-imipenem followed by daily oral *Lactobacillus GG* administration significantly decreased total colonic histology scores versus other treatment groups. *$p < 0.05$ versus other treatment groups. Each group consisted of six specific pathogen-free transgenic rats.

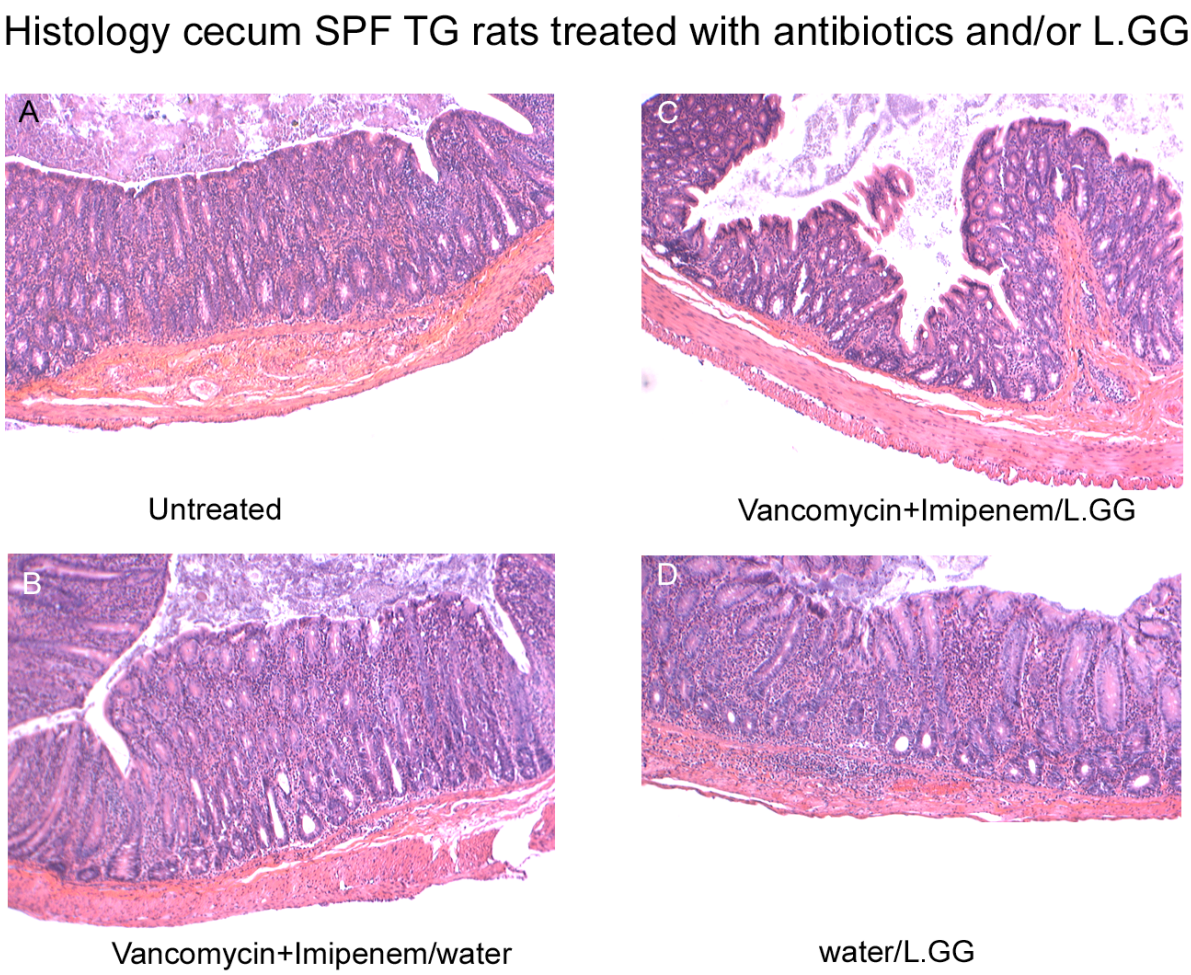

Figure 3. Representative photomicrographs of tissue sections (x40) from ceca of 16 weeks old specific pathogen-free transgenic rats which were treated with (**A**) water (**B**) two weeks oral vancomycin-imipenem followed by four weeks of water, (**C**) two weeks oral vancomycin-imipenem, followed by daily administration of *Lactobacillus GG* during four weeks (**D**) two weeks water, followed by four weeks oral *Lactobacillus GG*. Note the extensive mucosal and some submucosal inflammation as well as significant crypt hyperplasia in ceca in figures 2A-B and D. Only mild to modest mucosal inflammation was seen in transgenic rats treated with the combination of broad spectrum antibiotics followed by *Lactobacillus GG* (2C).

Cecal MPO values reflected the gross and histologic scores; only transgenic rats treated with antibiotics followed by *Lactobacillus GG* showed a significant reduction of cecal MPO (5.6 ± 1.0 U/g tissue) versus all other treatment groups, whereas MPO in cecal homogenates from transgenic rats treated with antibiotics alone (20.3 ± 5.6 U/g) or with *Lactobacillus GG* alone (10.2 ± 1.4 U/g) did not differ from untreated transgenic controls (13.3 ± 3.7 U/g) (figure 4). Cecal MPO from transgenic rats that had been fed Lactobacillus plantarum with or without antibiotic pre-treatment did not differ significantly from other treatment groups (data not shown).

Mucosal cytokines

Cecal homogenates from specific pathogen-free transgenic rats treated with antibiotics and/or *Lactobacillus GG* contained significantly less IL-1β than the other treatment groups (figure 5A). A similar pattern was shown for cecal TNF, although TNF concentrations were about 10-fold less than cecal IL-1β (figure 5B). Interestingly, IL-10 concentrations were significantly increased in cecal homogenates from antibiotics-*Lactobacillus GG* treated transgenic rats versus other treatment groups, whereas cecal TGF-β levels were no different among the groups (figure 5B).

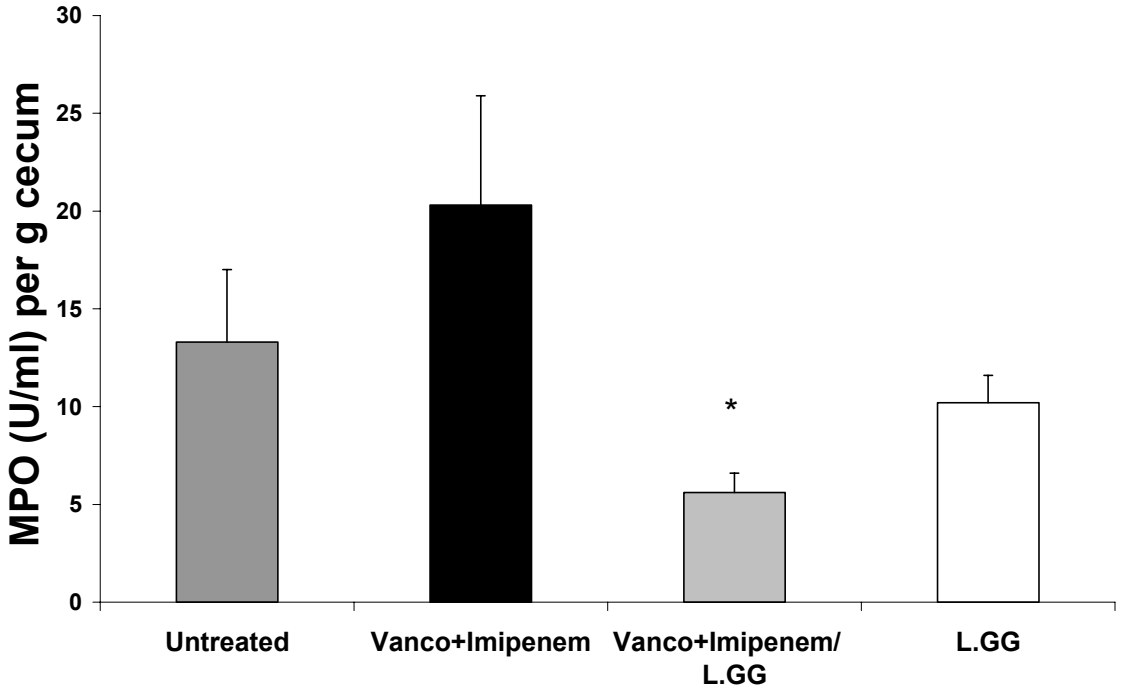

Figure 4. Cecal MPO concentrations in specific pathogen-free transgenic treated with two weeks oral broad spectrum antibiotics (Abs) or water followed by four weeks Lactobacillus GG or water. Values from untreated transgenic control rats are also given. Data represent mean MPO levels in Units per gram cecum. Error bars represent SEM. *p< 0.05 versus untreated controls.

Bacteriology of cecal contents

No differences were found in total bacterial counts of cecal contents between the antibiotic and/or *Lactobacillus GG* treatment groups in specific pathogen-free transgenic rats. Also concentrations of cecal anaerobic and aerobic bacteria as determined by culture on Brucella and SBA plates respectively were not significantly different (data not shown). Oral administration of *Lactobacillus GG* resulted in a 10-fold increase of *Lactobacillus* spp in cecal contents ($1.0 \pm 0.5 \times 10^7$ CFU/g with antibiotics alone versus $12 \pm 3 \times 10^7$ CFU/g cecal content in antibiotics-*Lactobacillus GG*-treated rats). However, cecal *Bacteroides* spp did not differ significantly among the treatment groups ($2.0 \pm 1.4 \times 10^{10}$ with antibiotics alone versus $2.7 \pm 0.3 \times 10^{10}$ CFU/g cecal content in the antibiotics-*Lactobacillus GG* -treated group).

In vitro inhibition of bacterial adherence

B. vulgatus was able to adhere to IEC-6 cells after an incubation of 3 hours. However, co-culture of 10^9 *B. vulgatus* and 10^9 *Lactobacillus GG* per well significantly reduced the amount of adherent *B. vulgatus*. No effect was noted with concentrations of 10^8/well (figure 6) or 10^7/well *Lactobacillus GG* (data not shown).

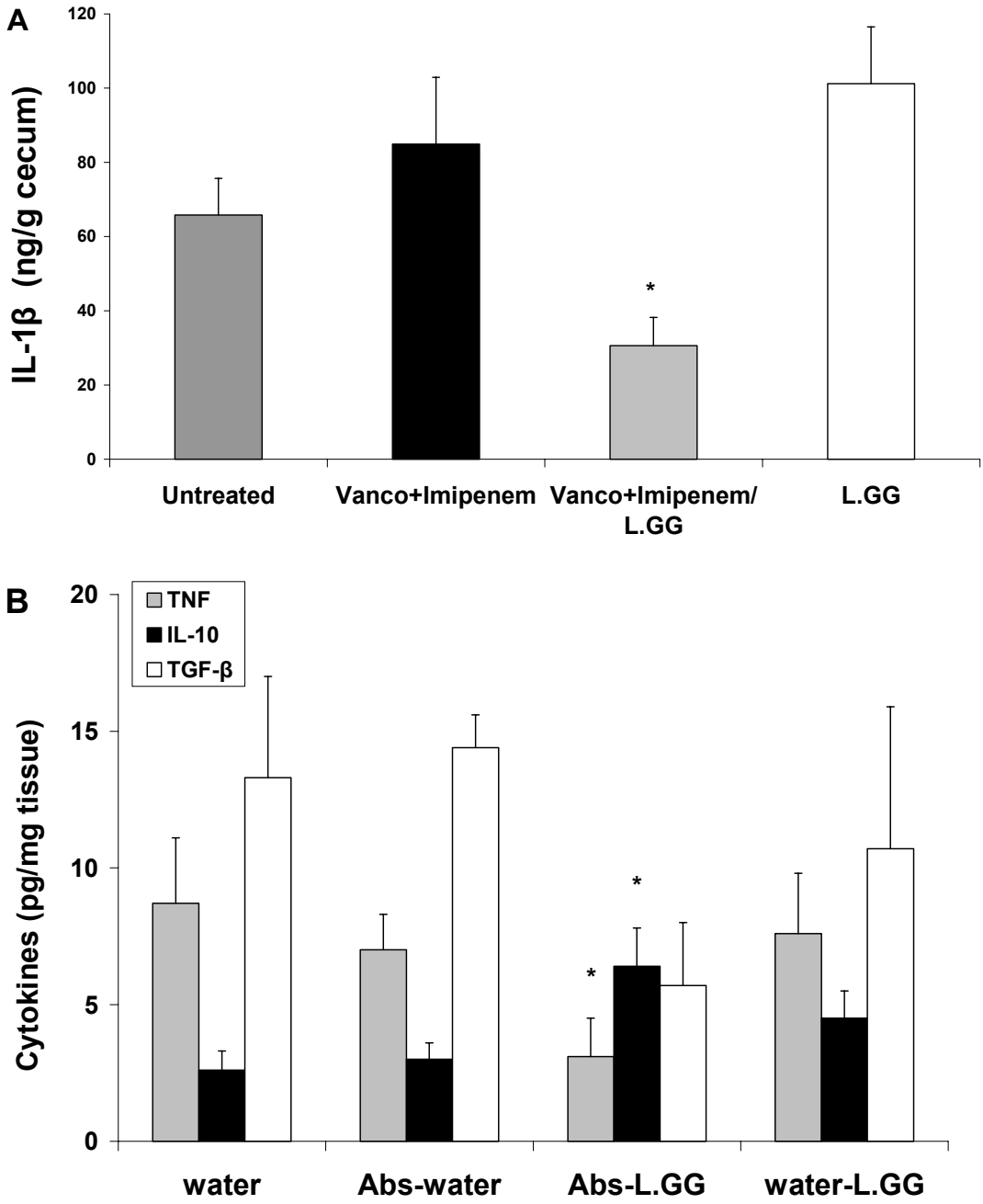

Figure 5. Cecal IL-1β (A) or TNF, IL-10 or TGF-β (B) concentrations (in pg/mg) from transgenic rats treated with two weeks oral broad spectrum antibiotics (Abs) or water followed by four weeks *Lactobacillus GG* or water as well as from untreated controls. Error bars indicate SEM. *p< 0.05 versus untreated controls. Combination of antibiotics followed by *Lactobacillus GG* significantly decreases cecal IL-1β and TNF, but also increases mucosal IL-10 concentrations.

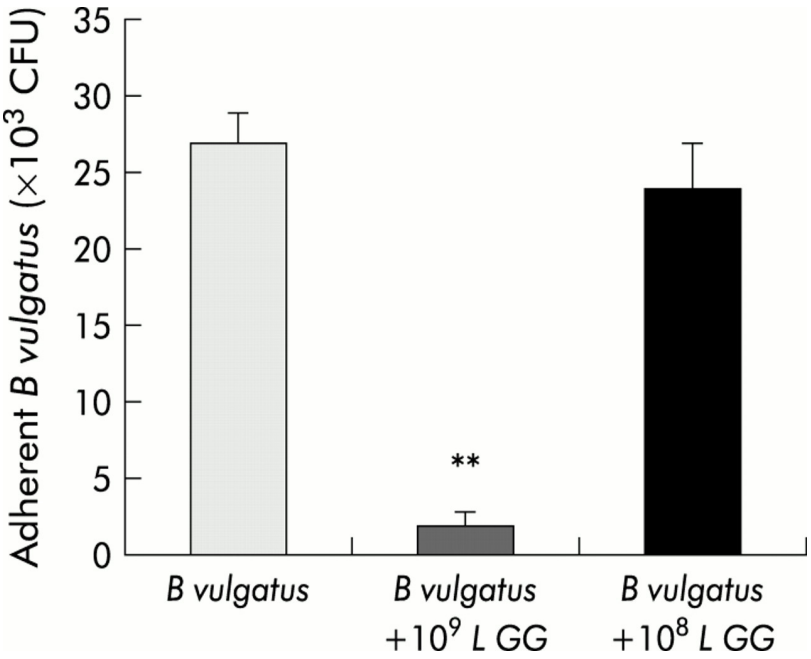

Figure 6. Dose-dependent inhibition of *in vitro* adherence of 10^9 CFU *B. vulgatus*/2 ml well to IEC-6 cells by *Lactobacillus GG*. Data represent concentrations (x 10^3 CFU) of adherent *B. vulgatus* after three hours incubation with IEC-6 monolayers in the presence or absence of 10^8 or 10^9 CFU *Lactobacillus GG* /2 ml well. Error bars represent SEM. ** $p< 0.01$ versus *B. vulgatus* control.

Discussion

Broad spectrum antibiotics can prevent as well as treat established colitis in specific pathogen-free HLA-B27 transgenic rats.[11] Similar results were obtained in other experimental colitis models in rats and mice, emphasizing the important role of intestinal bacteria for the induction and perpetuation of chronic intestinal inflammation.[11,26-27] Oral vancomycin-imipenem treatment resulted in undetectable levels of *Bacteroides* spp, which are capable of selectively inducing colitis in HLA-B27 transgenic rats.[10,13]

We found that colitis relapsed within four weeks of stopping vancomycin-imipenem treatment of established colitis in specific pathogen-free transgenic rats, resulting in disease relapse within four weeks with gross and histologic scores, MPO and proinflammatory cytokine levels nearly identical with untreated controls. This finding correlated with the re-appearance of luminal *Bacteroides* spp, since cecal *Bacteroides* concentrations were not significantly different in antibiotic-treated rats versus untreated transgenic controls. These findings are in agreement with clinical observations in Crohn's disease, which recurs after stopping antibiotic treatment.[28] These observations can be explained by the reappearance of disease-inducing intestinal bacteria in genetically susceptible hosts.

A clinically relevant finding of our study was that oral *Lactobacillus GG* treatment partially prevented relapse of colitis after antibiotic treatment, whereas *Lactobacillus GG* administration alone failed to show a beneficial effect. This conclusion was supported by significantly decreased gross cecal inflammation and colonic histology scores, as well as a significant decrease of nonspecific inflammatory markers and cytokines, such as cecal MPO, IL-1 and TNF in antibiotic-*Lactobacillus GG*-treated specific pathogen-free transgenic rats versus other treatment groups. This is the first report of an interactive effect of antibiotic and probiotic

therapy in experimental colitis. These results are in agreement with Gionchetti et al, in which relapsing pouchitis was prevented in 85% of refractory pouchitis with an oral probiotic cocktail VSL#3, which includes four strains of *Lactobacilli*, three *Bifidobacteria* species and *Streptococcus salivarius* subsp. *thermophilus* after the broad spectrum antibiotic rifaximin was stopped.[20] Similarly, prospective treatment with rifaximin followed by VSL#3 could also prevent endoscopic post-operative recurrence of Crohn's disease.[29]

Another major finding in our study was the specificity of prevention of relapsing colitis by various *Lactobacillus* species as demonstrated by the lack of efficacy of *Lactobacillus plantarum* in preventing colitis relapse in specific pathogen-free transgenic rats after antibiotic treatment. However, this result is in contrast with our previous reports that oral *Lactobacillus plantarum* attenuated colitis in IL-10$^{-/-}$ mice,[20] whereas *Lactobacillus GG* had no effect.[30] A preventive effect of *Lactobacillus plantarum* was also shown in methotrexate-induced enterocolitis in rats.[31] These contrasting responses suggest that different *Lactobacilli* may have variable host specificity or different efficacy in various inflammatory conditions, indicating that not all probiotics are equally protective in chronic experimental colitis. The concept of selective dependency of beneficial effects of a probiotic bacterial species on the species and genetic background of the host is similar to that of disease-inducing bacteria.[10, 13, 32-33]

Other studies have reported primary beneficial effects of probiotics in the treatment or prevention of human or experimental intestinal inflammation. VSL#3 was able to treat established colitis in IL-10$^{-/-}$ mice.[25] The same cocktail has also shown efficacy in an open-labeled study on the maintenance therapy of ulcerative colitis.[34] *Lactobacillus GG* can prevent relapsing *Clostridium difficile* diarrhea,[19] traveler's diarrhea[35] and rotavirus infections in children.[36] The non-pathogenic *E. coli* strain Nissle was shown to be as effective as low dose mesalamine in maintaining remission of ulcerative colitis.[37-38]

The protective mechanisms of probiotic bacteria are still relatively unknown, with postulated effects on luminal microecology, mucosal barrier function and immunoregulation. In our study there was no decrease in the number of luminal *Bacteroides* spp, raising the possibility that some of the protective effects of antibiotics-*Lactobacillus GG* could be explained by immunomodulation. Not only does this therapy result in decreased cecal proinflammatory cytokines IL-1β and TNF, but it also increased mucosal IL-10. The lack of stimulation of mucosal IL-10 by *Lactobacillus GG* or antibiotics alone indicate that neither treatment is responsible for induction of IL-10. It is possible that combination antibiotics and probiotics alters the balance of enteric bacterial components, leading to altered mucosal cytokine profiles. IL-10 is an immunoregulatory cytokine, mainly produced by macrophages, dendritic cells and T_R1 lymphocytes. However, we recently reported that in cecal bacterial lysate-stimulated mesenteric lymph node cells of HLA-B27 transgenic rats as well as their non-transgenic littermates IL-10 is primarily produced by B cells.[39] *In vitro*, lysates of VSL#3 stimulates IL-10 production and diminishes TNF secretion by rat splenocytes stimulated with cecal bacteria.[40] In preliminary data, it was demonstrated that DNA extracts of VSL#3 induce IL-10 and exert protective immune responses.[41] IL-10 can inhibit antigen-specific proliferation and cytokine secretion by T_H1 lymphocytes and has downregulatory effects on macrophages and dendritic cells, such as suppression of activation and IL-12 production.[42-43] IL-10 can also prevent IFN-γ-induced disruption of colonic epithelial barriers.[44] Defective *in vivo* production of IL-10 results in spontaneous colitis in IL-10$^{-/-}$ mice in the presence of normal intestinal bacteria.[7,45] Furthermore, oral administration of *Lactococcus lactis*, engineered to produce high colonic IL-10 levels, prevents the onset of colitis in IL-10$^{-/-}$ mice as well as in DSS-induced colitis.[46] Thus, stimulation of colonic IL-10 production by the combination of antibiotics use and *Lactobacillus GG*

colonization could have a variety of beneficial effects on mucosal immunoregulation and barrier function.

Increased mucosal IL-10 production and decreased levels of the proinflammatory cytokines IL-1β and TNF by the combination of antibiotics and *Lactobacillus GG* in our study are in agreement with findings of Ulisse et al, who showed increased IL-10 as well as decreased TNF and IFN-γ in pouch biopsies of refractory pouchitis patients treated with VSL#3 after antibiotic pretreatment.[47] Similar to our findings, these authors found that tissue IL-10 levels were unchanged in inflamed pouches and were not affected by prior antibiotic treatment.[47] However, in IL-10$^{-/-}$ mice the probiotic cocktail VSL#3 treated colitis with a concomitant decrease of mucosal IFN-γ and TNF secretion,[21] indicating that beneficial effects of probiotics in colitis can be mediated by factors other than IL-10 induction.

Several non-immune protective mechanisms have been described for probiotic bacterial species. These organisms can exert growth inhibitory and bactericidal activities on enterotoxigenic *E. coli.*[48] However, in our study cecal concentrations of the disease-inducing *Bacteroides* spp were not significantly decreased. Another potential protective non-immune mechanism involves inhibition of epithelial adherence of disease-inducing organisms. Intestinal bacteria compete with other organisms for a limited number of epithelial receptors. Increased amounts of colonic mucosal-associated bacteria were found in IL-10$^{-/-}$ mice[28] well as in mucosal biopsies from IBD patients.[49] Madsen et al also reported a relative lack of adherent *Lactobacilli* in IL-10$^{-/-}$ and a restoration to normal levels after probiotic treatment.[19, 21] Our *in vitro* co-culture studies demonstrate that *Lactobacillus GG* only at high concentrations of 10^9 CFU/ml decreased adherence of *B. vulgatus* to rat IEC-6 cells, with no effects at lower doses. This result is in agreement with *in vitro* studies by Madsen[21] and Mack[25] in which probiotic bacteria prevented the epithelial adherence of *Salmonella dublin* or enteropathogenic *E. coli*. However, in our study *in vivo* co-association of *B. vulgatus* and *Lactobacillus GG* did not prevent colitis in gnotobiotic transgenic rats. This lack of effect by *Lactobacillus GG* in the dual association model could be explained by the overwhelming colonization capacity of the disease-inducing *B. vulgatus* versus *Lactobacillus GG*, which were 100 fold more frequent than *Lactobacillus GG*. We suspect that the lack of effect by *Lactobacillus GG* in the dual association model could be explained by the overwhelming colonization capacity of the disease-inducing *B. vulgatus* versus *Lactobacillus GG*, which were 100 fold more frequent than *Lactobacillus GG*. It is possible that at this relatively low *in vivo* luminal concentration *Lactobacillus GG* would not be able to mediate protection by inhibiting epithelial adherence of *B. vulgatus*, since equal concentrations of *Lactobacillus GG* were required to interfere with *B. vulgatus* epithelial adherence. Based on these findings we postulate that protective effects by probiotics in specific pathogen-free transgenic rats could only be achieved by pretreatment of the rats with vancomycin-imipenem, which would result in a significant decrease of *B. vulgatus* before *Lactobacillus GG* treatment.[11] However, despite the prevention of colitis relapse by the combination treatment of vancomycin-imipenem followed by *Lactobacillus GG* this treatment did not significantly reduce luminal *Bacteroides* spp, even though increased cecal *Lactobacilli* spp was shown by cecal culture. Taken together, reduction of luminal *Bacteroides* spp could not explain the protective effects of broad spectrum antibiotics followed by *Lactobacillus GG*. Prevention of colitis relapse as well as immunomodulatory effects were shown only with the combination treatment of vancomycin-imipenem followed by *Lactobacillus GG*. It is possible that the combination treatment promoted synergistic interaction of *Lactobacillus GG* with other endogenous probiotic species, resulting in disease protection. The absence of these potential beneficial interactions with other intestinal protective bacteria in gnotobiotic transgenic rats co-associated with *Lactobacillus GG* and *B. vulgatus* could also

explain the lack of efficacy of *Lactobacillus GG* in prevention of colitis. Alternatively, lack of exposure to lactic acid bacteria early in life in these gnotobiotic rats may prevent stimulation of protective responses by *Lactobacillus GG* at an older age.

In summary, oral *Lactobacillus GG* can prevent relapse of colitis in specific pathogen-free B27 transgenic rats after antibiotics are stopped, whereas oral administration of *Lactobacillus plantarum* did not have any effect, demonstrating the selective protective effects of two *Lactobacillus* species. The beneficial *in vivo* effects of *Lactobacillus GG* following antibiotics are possibly the result of enhanced IL-10 production. This is the first study in experimental colitis in which antibiotics and probiotics display synergistic *in vivo* effects. Restoring the microbial balance between detrimental and protective luminal bacteria by combining antibiotic and probiotic approaches may be the most physiologic approach to treat IBD and may alter the natural history of these chronic relapsing diseases.

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Administration of the prebiotic combination inulin and oligofructose prevents colitis in HLA-B27 transgenic rats

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Frank Hoentjen[1,2], Gjalt W. Welling[3], Hermie J. M. Harmsen[3]
Xiaoyin Zhang[1], Jennifer Snart[4], Gerald W. Tannock[4]
Kelvin Lien[4], Maryla Lupicki[5] and Levinus A Dieleman[1,5]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

[3]Department of Medical Microbiology
University of Groningen, The Netherlands

[4]Agricultural, Food and Nutritional Science, and
[5]Department of Gastroenterology
University of Alberta, Edmonton, Canada

Submitted

Abstract

Introduction and aims

HLA-B27 transgenic rats develop spontaneous colitis when housed under specific pathogen-free conditions but germ-free rats remain disease-free, emphasizing a role for intestinal bacteria in the pathogenesis of the disease. Prebiotics are nutrients that affect the host by stimulating growth and/or activity of potentially health promoting (probiotic) bacteria. The aims of this study were to investigate whether prebiotics can prevent colitis in specific pathogen-free HLA-B27 rats, and secondly, to explore mechanisms of protection.

Methods

Specific pathogen-free HLA-B27 transgenic rats received orally the prebiotic combination inulin/oligofructose, or not, prior to the development of clinically detectable colitis. After seven weeks, the rats were killed, and cecal and colonic tissues were collected for gross cecal scores, histological inflammatory scores (scale 0-4), and mucosal cytokine measurement. Cecal and colonic contents were collected for PCR-DGGE analysis of the gut microbiota, and enumeration of selected bacterial populations by FISH.

Results

Prebiotic treatment significantly decreased gross cecal scores and inflammatory histological scores in the cecum and colon. Prebiotic treatment also decreased cecal IL-1β, but increased cecal TGF-β concentrations. Inulin/oligofructose altered the cecal and colonic PCR-DGGE profiles, and FISH analysis showed significant increases in cecal *Lactobacillus* and *Bifidobacterium* populations after prebiotic treatment compared to water-treated rats.

Conclusions

The prebiotic combination inulin/oligofructose partially prevented colitis in HLA-B27 transgenic rats, which was associated with alterations to the gut microbiota, decreased tissue proinflammatory cytokines and increased immunomodulatory molecules. These results show promise for prebiotics as primary or adjuvant maintenance therapy for chronic inflammatory bowel diseases.

Introduction

The role of the gut microbiota in the pathogenesis of IBD is well recognized.[1,2] These diseases typically occur at sites with the highest concentrations of intestinal bacteria, such as the colon and terminal ileum. The role of intestinal bacteria and the genetic background of the host in the initiation and perpetuation of chronic intestinal inflammation are most convincingly demonstrated in rodent models of chronic intestinal inflammation.[3] In these models, genetically susceptible hosts develop spontaneous colitis in the presence of intestinal bacteria, but not in the germ-free state.[3]

One example of a well-characterized model of intestinal inflammation is the HLA-B27 transgenic rat, which develops colitis, gastritis, and arthritis in a specific pathogen-free environment.[4] However, germ-free transgenic rats, as well as non-transgenic rats colonized with specific pathogen-free bacteria, do not show evidence of colitis, gastritis, and arthritis.[5] Colitis and gastroduodenal inflammation in transgenic rats can be prevented with broad spectrum as well as selective antibiotics.[6] Moreover, cecal bacterial overgrowth within an experimental blind loops exacerbates colitis, whereas bypass of the cecum attenuates disease in this model.[7] *B. vulgatus* preferentially induces colitis in transgenic rats after monoassociation for four weeks, whereas monoassociation with *E. coli* does not cause disease.[8] These findings indicate that although bacteria are crucial in the pathogenesis of colitis, not all bacteria are equal in their capacity to induce intestinal inflammation.

Probiotics are "defined, live microorganisms administered in adequate amounts which confer a beneficial physiological effect on the host." [9] We and others have shown that certain probiotic bacteria such as *Lactobacillus* species have protective activities in several experimental models of chronic intestinal inflammation, including specific pathogen-free HLA-B27 transgenic rats and specific pathogen-free IL-10$^{-/-}$ mice.[10,11] Probiotic cocktails have also proven efficacy in ulcerative colitis[12] and refractory pouchitis.[13]

Prebiotics are non-digestible polysaccharides or oligosaccharides that affect the host by selectively stimulating growth and/or activity of a limited number of potentially health promoting (probiotic) bacteria.[14] Previous studies have demonstrated that the prebiotics inulin and oligofructose selectively stimulate the growth of endogenous *Bifidobacteria* or *Lactobacilli*.[15,16] To date, the efficacy of prebiotics as monotherapy has been limited to induced models of colitis.[17,18] However, as probiotics were effective in specific pathogen-free HLA-B27 transgenic rats[11] we designed this study to determine if prebiotics would have a similar degree of protection in this model. In addition, the role of immune factors, cecal and colonic SCFA, and the composition of the intestinal microbiota were studied as possible mechanisms of protection. In this study, we demonstrate that the combination of inulin and oligofructose partially prevented colitis. This beneficial effect was associated with alterations to the gut microbiota, a decrease in cecal proinflammatory cytokines, and increased immunoregulatory cytokines. These results show promise for prebiotics as primary or adjuvant maintenance therapy for chronic inflammatory bowel diseases.

Materials and methods

Animals

HLA-B27 transgenic rats (the 33-3 line on the F344 background) and their non-transgenic littermates were originally obtained from Dr. Joel D. Taurog, University of Texas Southwestern

Medical Center. Rats were born and maintained in specific pathogen-free housing conditions at the University of North Carolina, Chapel Hill. Presence or absence of the HLA-B27 transgene was determined by PCR using DNA isolated from tail clippings as described previously.[19] All studies were approved by University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Experimental design

Seven week old specific pathogen-free HLA-B27 transgenic rats, prior to the development of histologic inflammation, were divided into two groups of six rats each. The first group was administered a combination of chicory inulin and oligofructose in a mixture of 1:1 (Raftilose Synergy1, Orafti, Tienen, Belgium) in their drinking water at a dose of 7.5 g/kg bodyweight, whereas the control group received no addition. The amount of prebiotics consumed by each rat per day was calculated by measured consumption of 30 ml of water per day. Rats were killed after seven weeks. At necropsy cecal and colonic tissue and content were snap-frozen for subsequent cytokine analysis and microbiological and SCFA assessment. Mesenteric lymph node cells were collected for cell cultures.

Gross cecal scores

The cecum and colon were inspected grossly in a blinded fashion for evidence of intestinal wall thickness and scored on a scale ranging from 0 to 4, using a previously validated system.[11,20]

Histology

Colons and ceca were fixed and stained as previously described.[11,20] A validated histologic inflammatory score ranging from 0 to 4 was used for blinded evaluation of cecal and colonic inflammation.[20]

Preparation of cecal bacterial lysates

Cecal bacterial lysates were prepared as described previously.[19] Briefly, cecal contents from several specific pathogen-free HLA-B27 non-transgenic rats were homogenized and the supernatant was filtered through a 0.45 μm syringe filter. Sterility was confirmed by aerobic and anaerobic culture.

Mesenteric lymph node cell cultures

Mesenteric lymph nodes were removed from water- and prebiotic-treated HLA-B27 transgenic rats, and single cell suspensions were prepared by gentle teasing, as previously described.[19] Mesenteric lymph node cells were washed twice and $4x10^5$ cells were cultured in 96 well flat bottom microplates (Costar 3595), in 0.2 ml complete medium, as described previously.[19] Cells were stimulated with the optimal concentration of 50 μg/ml cecal bacterial lysate, as described previously.[19] Culture supernatants were collected after three days and stored at -20°C.

Cytokine measurements in mesenteric lymph node cell cultures

Cytokines in cell culture supernatants were measured by ELISA using unlabeled capture antibodies and biotin-labeled detection antibodies, followed by horse-radish peroxidase labeled Streptavidin.[19] The concentration of each cytokine was determined by comparison to a standard curve generated using recombinant proteins. For IFN-γ, we used unlabeled polyclonal anti-IFN-γ antibody and biotin-labeled monoclonal anti-IFN-γ antibody (clone DB-1) (Biosource International, Camarillo, CA). For IL-10 we used unlabeled monoclonal anti-rat IL-10 antibody

(clone A5-7) and biotin-labeled monoclonal anti-rat IL-10 antibody (clone A5-6) (BD Biosciences Pharmingen, San Diego, CA).

Cecal cytokine analysis

Cecal cytokines were measured as described previously.[11] Briefly, frozen cecal tissues were thawed and lysed in PBS containing a cocktail of protease inhibitors.[21] Tissue was then homogenized after which the homogenate was assayed for IL-1β, IL-10 and TGF-β, as described.[11] IL-1β was measured by ELISA according to the manufacturer's instructions (National Institute for Biological Standards and Controls, South Mimms, UK). IL-10 was measured as described in the previous paragraph. TGF-β concentrations were measured after acidification and neutralization according to the manufacturer's instructions using a TGF-β specific ELISA (Promega, Madison, WI, USA).

SCFA analysis in cecal contents

Cecal or colonic content (100 mg portions) was combined with 400 µl water and 100 µl of 25% phosphoric acid in a 2 mL microcentrifuge tube, mixed thoroughly, and immediately frozen. At the time of analysis, samples were thawed and 100 µl of internal standard (4-methyl-n-valeric acid at 50 µmol/ml) was added. The samples were centrifuged to obtain a clear supernatant that was placed into 1.8 mL GC vials for analysis. SCFA separation was performed using a Varian 3400 gas chromatograph equipped with a Varian 8200CX autosampler. Approximately 1 µl was injected onto a Stabilwax-DA GC capillary column (30m x 0.53mm ID: Restek Corp., Bellefonte PA) with an injector split ratio of 20:1. Injector and detector temperatures are set at 170°C and 190°C, respectively. Column temperature was increased from 120°C to 170°C at 10°C/min and held for 5 minutes. Data acquisition and integration were performed using an Schmadzu Class-VP Chromatography Laboratory Automated Software System.

PCR-DGGE from cecal and colonic contents

In order to detect changes in the composition of the large bowel microbiota of rats, we utilized PCR-DGGE. DNA was extracted from cecal and colonic samples by preparing a 1/10 (wt/vol) homogenate in PBS (pH 7.0). Samples were vortexed and stored at -80°C until further use. DNA extraction followed a previously described method.[22] Amplification of total bacterial community DNA was carried out by targeting 16S rDNA sequences using universal bacterial primers HDA1-GC and HDA-2 and using a procedure described previously.[22] The PCR products were checked by electrophoresis through a 2% agarose gel, stained with ethidium bromide (5 µg/ml) and viewed by UV transillumination. PCR amplicons were analyzed by DGGE. This was performed with a DCode apparatus (Bio-Rad, Hercules, CA), using a 6% polyacrylamide gel with a 30 to 55% gradient of 7.0 M urea and 40% (vol/vol) formamide that increased in the direction of the electrophoresis. Electrophoresis was carried out in 1× TAE buffer at 130 V and 60°C for 4 hours. Gels were stained with ethidium bromide solution (5 µg/ml) for 20 min, washed with deionised water for 20 min, and viewed by UV transillumination. DNA fragments of interest were cut from the polyacrylamide gel using a sterile scalpel blade after the stained gel had been photographed. DNA was eluted from the gel, cloned and sequenced as described previously.[23] Sequences were compared to those in the GenBank database using the BLASTn algorithm in order to identify the bacterial species from which the DNA sequence originated.[24]

Fluorescent *in-situ* hybridization

FISH was carried out as described previously.[25] Briefly, 100 mg of cecal or colonic content was diluted, homogenized, fixed overnight in paraformaldehyde at 4ºC, and then stored at -80ºC until analysis. Total cell numbers were counted by DAPI staining, while probe Erec482 was used for the detection of cells from the *Clostridium*-group XIVa,[26] probe BAC303 for the detection of the *Bacteroides/Prevotella* genera,[27] probe Bif164 for the detection of the genus *Bifidobacterium*[28] and probe Lab158 to detect *Lactobacilli* and *Enterococci*.[29] The samples hybridized overnight at 50ºC in a dark humid chamber.[30] After hybridization, the slides were washed, air dried, mounted, and the fluorescent cells in the samples were counted automatically with a Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany).[31] However, when the cell number was lower than $4x10^8$ cells/g wet weight, the cells were counted visually using an Olympus BH2 epifluorescence microscope.[30] All results are stated as bacterial counts/g luminal content, thus normalized for the weight of luminal contents used during FISH analysis. Oligonucleotides probes were synthesized and purified by Eurogentec (Seraing, Belgium).

Statistical analysis

Statistics were performed using the non-paired Student's t test for comparison between groups. Data are expressed as mean ± SEM, and a two-tailed p value of < 0.05 was considered statistically significant.

Results

Gross cecal scores and histology

Daily water consumption did not differ significantly between the experimental groups (data not shown). Oral administration of the prebiotic combination inulin and oligofructose to HLA-B27 transgenic rats partially prevented the development of colitis. Gross cecal scores were significantly decreased in prebiotic-treated rats (figure 1), whereas water-treated animals showed severe cecal macroscopic inflammation ($P < 0.005$). This beneficial effect of prebiotic treatment was also reflected by significantly lower histological scores for cecum ($P < 0.01$) and colon ($P < 0.005$) (figure 2) in prebiotic-treated versus untreated transgenic rats. Severe mucosal and submucosal inflammation with significant crypt hyperplasia was seen in untreated transgenic rats (figure 3A), whereas prebiotic treatment resulted in only modest mucosal inflammation (figure 3B). Interestingly, all water-treated rats developed arthritis in one or more joints, whereas only one out six rats in the prebiotic group showed arthritis (data not shown).

Mucosal cytokine profile

Cecal IL-1β concentrations were decreased after prebiotic treatment ($P < 0.05$ versus water controls; figure 4A). In contrast, prebiotics increased cecal TGF-β secretion ($P < 0.05$ versus water controls; figure 4B), whereas cecal IL-10 production did not significantly differ between the groups (data not shown).

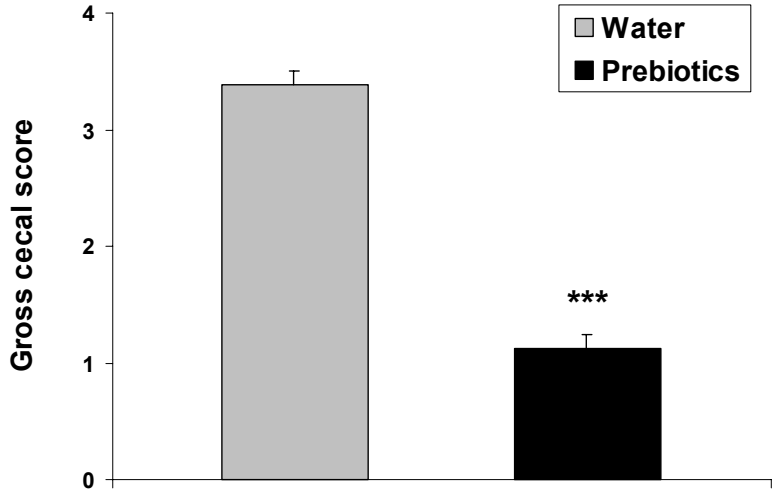

Figure 1. Decreased gross cecal scores (0-4)after prebiotic treatment. Specific pathogen-free transgenic rats were treated for seven weeks with prebiotics inulin and oligofructose (mixture 1:1) or water prior to the development of histologic inflammation. Each group consisted of six rats. Values are presented as mean ± SEM, *** $P < 0.005$ versus untreated transgenic rats.

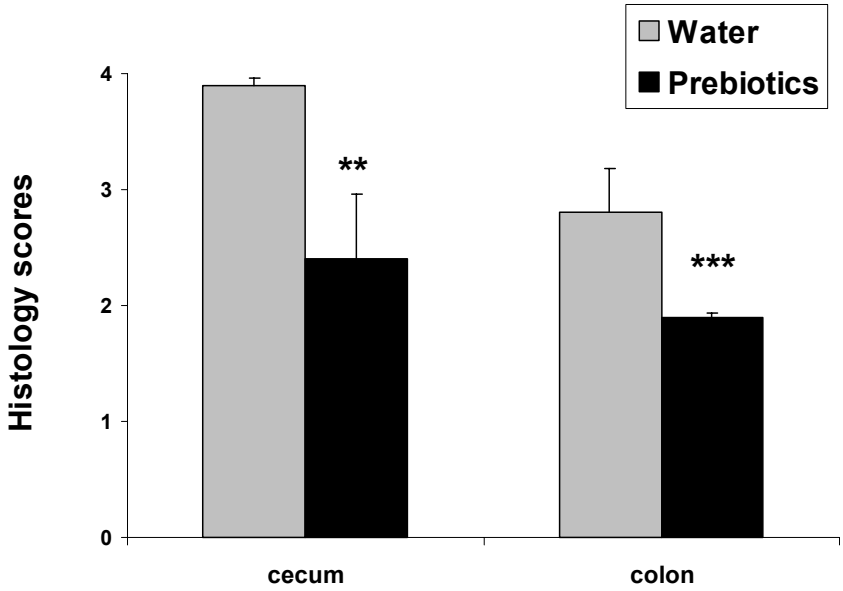

Figure 2. Decreased histologic scores after prebiotic treatment. Blinded microscopic inflammation scores from ceca and colons in specific pathogen-free transgenic rats were treated for seven weeks with prebiotics inulin and oligofructose (mixture 1:1) or water prior to the development of histologic inflammation. Each group consisted of six rats. Values are presented as mean ± SEM, ** $P < 0.01$, *** $P < 0.005$ versus untreated transgenic rats.

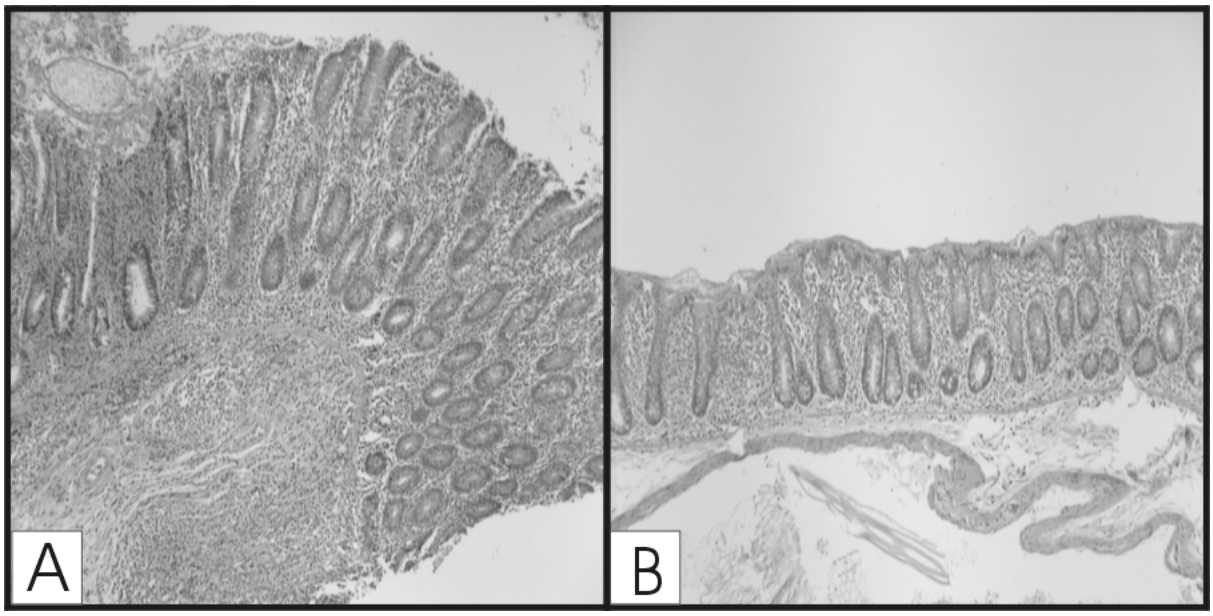

Figure 3. Decreased cecal inflammation in transgenic rats after prebiotic treatment. Representative photomicrographs of tissue sections (x40) from ceca of 14 weeks old specific pathogen-free transgenic rats were treated for seven weeks with water (A) or the prebiotics inulin and oligofructose (mixture 1:1) (B) prior to the development of histologic inflammation. Note the extensive mucosal and submucosal inflammation as well as significant crypt hyperplasia in ceca from water-treated transgenic rats (A). Only modest mucosal inflammation was seen in transgenic rats treated with the prebiotic combination (B).

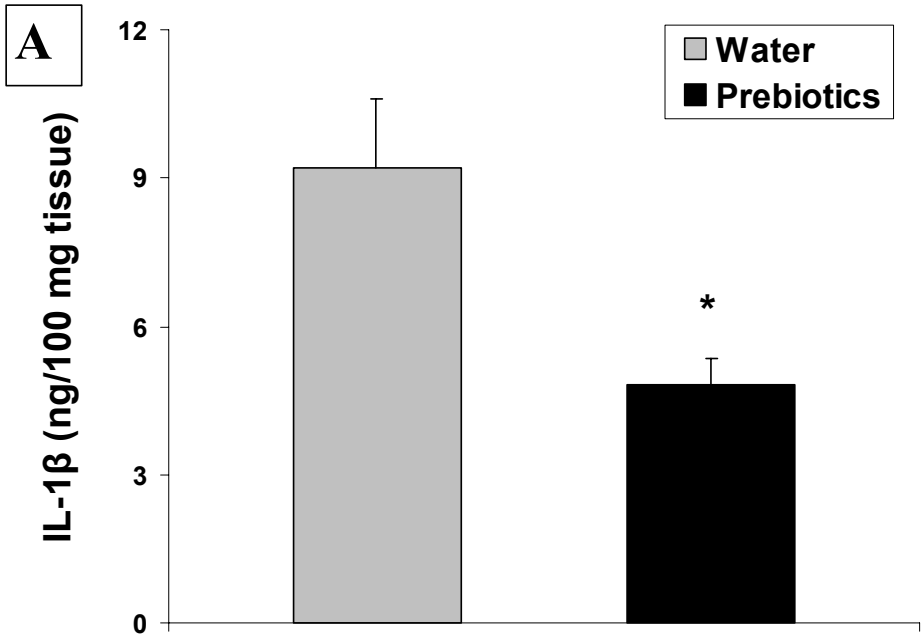

Figure 4A. Altered cecal cytokine profile after prebiotic treatment. Cecal IL-1β concentrations (ng/100 mg tissue) (A) from transgenic rats treated with either water or oral prebiotics prior to the development of histologic inflammation. Each group consisted of six rats. Values are presented as mean ± SEM, * $P < 0.05$ versus untreated transgenic rats.

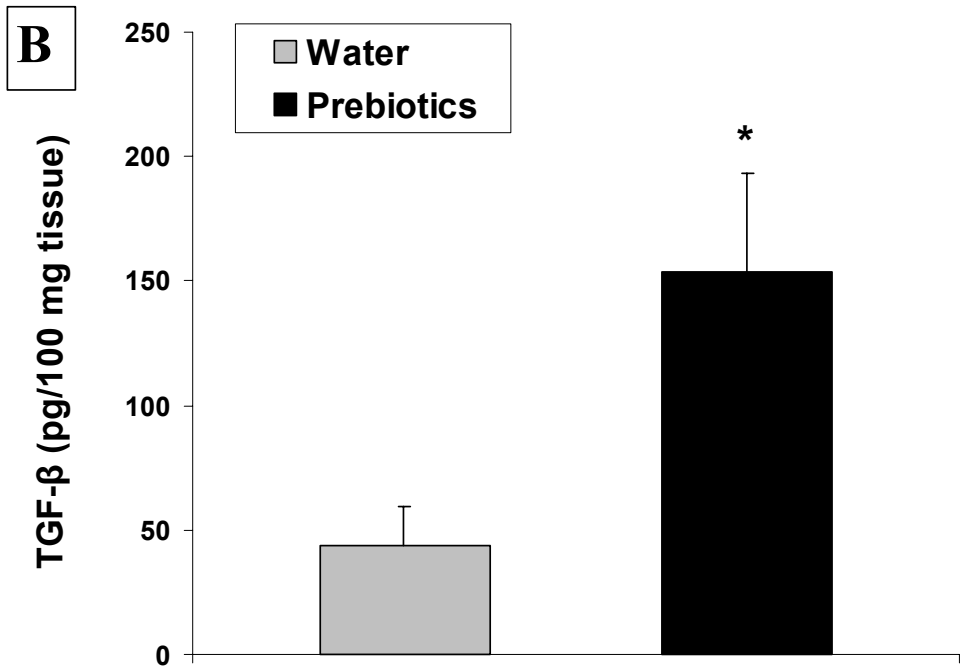

Figure 4B. Altered cecal cytokine profile after prebiotic treatment. TGF-β concentrations (pg/100 mg tissue) (B) from transgenic rats treated with either water or oral prebiotics prior to the development of histologic inflammation. Each group consisted of six rats. Values are presented as mean ± SEM, * $P < 0.05$ versus untreated transgenic rats.

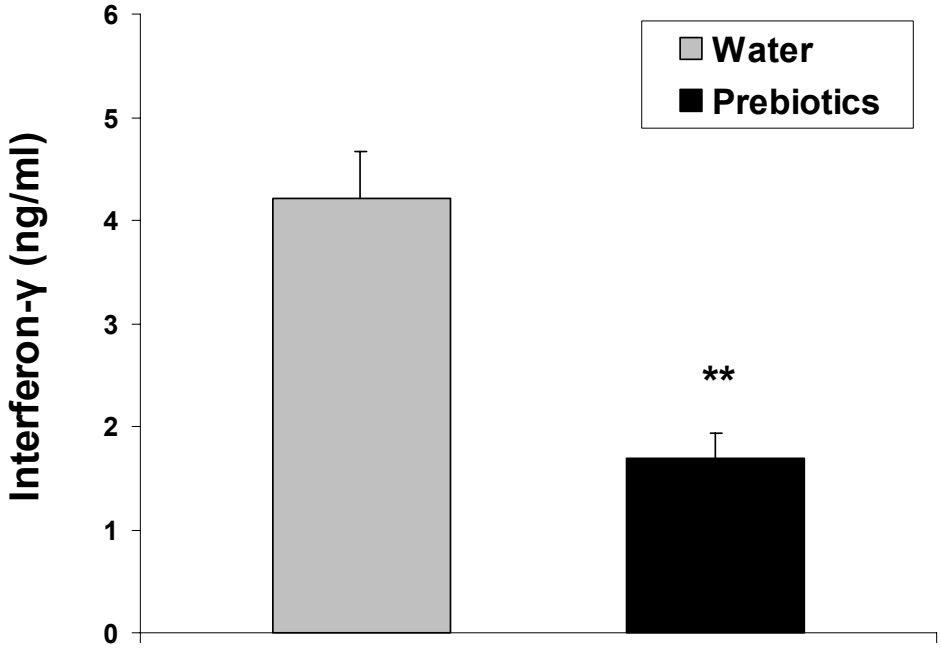

Figure 5. Decreased interferon-γ secretion after prebiotic treatment. Specific pathogen-free transgenic rats were treated with either water or prebiotics prior to the development of histologic inflammation. After seven weeks, rats were killed and mesenteric lymph nodes were collected. Mesenteric lymph node cells were isolated and cultured in the presence of cecal bacterial lysate at 50 μg/ml protein concentration. Interferon-γ concentrations (ng/ml) were measured in the triplicate supernatants by ELISA. Each group consisted of six rats. Values are presented as mean ± SEM, ** $P < 0.01$ versus untreated transgenic rats.

Mesenteric lymph node cell cytokine responses

Stimulation with cecal bacterial lysate induced higher interferon-γ responses in mesenteric lymph node cells from untreated transgenic rats compared to prebiotic-treated animals ($P < 0.01$; figure 5). No differences in IL-10 production were found for cecal bacterial lysate-stimulated mesenteric lymph node cells from prebiotic- versus untreated transgenic rats (data not shown).

PCR-DGGE profiles and FISH enumeration of cecal and colonic bacteria

A relatively simple microbiota profile was detected in cecal and colonic contents of the HLA-B27 rats that had been administered the prebiotic preparation (figure 6). Most strikingly, the profiles of untreated transgenic rats lacked a DNA fragment originating from *Bifidobacterium animalis* (figure 6; as indicated by arrow) that was present in prebiotic-treated transgenic rat profiles.

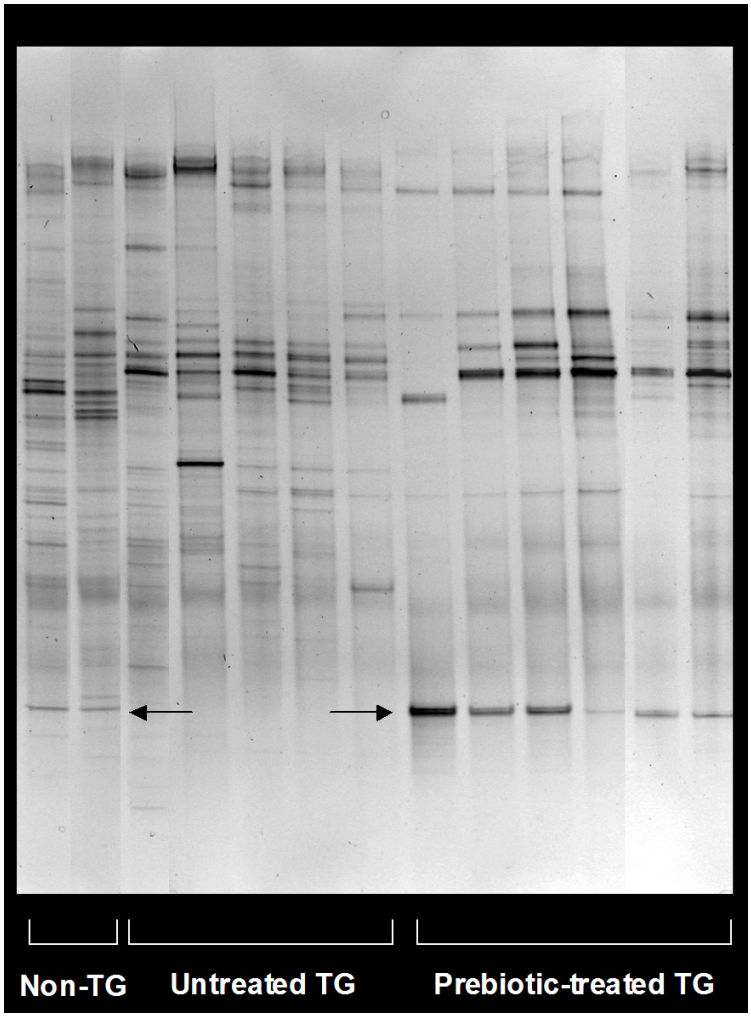

Figure 6. Altered PCR-DGGE profiles result from prebiotic treatment. Specific pathogen-free transgenic rats were treated for seven weeks with either water or prebiotics prior to the development of histologic inflammation. At necropsy bacterial DNA was extracted from cecal and colonic contents and PCR-DGGE was performed as described in the materials and methods section. The gel shows microbiota profiles generated from cecal contents derived from water-treated transgenic rats and inulin/oligofructose treated rats. Each lane represents a single sample. Arrows indicate 16S rRNA gene fragments originating from *Bifidobacterium animalis* (>97% identity). Non-TG; non-transgenic, TG; transgenic

Bacterial Group (probe or dye) Treatment	Total Bacterial Count (Dapi) x 10^9	*Clostridium* Group XIVa (Erec482) x 10^9	*Bacteroides/ Prevotella* (Bac303) x 10^8	*Bifidobacteria* (Bif164) x 10^7	*Lactobacilli/ enterococci* (Lab158) x 10^7
Water	4.4 ± 1.2	2.6 ± 0.8	5.1 ± 3.0	8.8 ± 3.1	4.0 ± 0.8
Inulin/ Oligofructose	12.3 ± 3.4	6.2 ± 1.9	8.2 ± 2.6	88.9 ± 32.8[a]	24 ± 0.6[a]

Table 1. Quantitative bacteriology of cecal contents of HLA-B27 transgenic rats by FISH seven weeks after treatment with either water or inulin/oligofructose prior to the development of histologic inflammation. Values represent mean ± SEM bacterial counts per gram cecal stool. [a] $P < 0.05$ versus untreated group

This suggested that the bifidobacterial population had been influenced by prebiotic consumption. This was confirmed by enumeration of *Bifidobacteria* by FISH (table 1). Additionally, FISH analysis showed an increase in *Lactobacillus* numbers in animals administered the prebiotic preparation (table 1). The microbiota profile in colonic contents did not differ from that of the cecum (data not shown).

Cecal SCFA concentrations

Analysis for the SCFA concentrations in the luminal contents of the cecum indicate that there were no significant differences between untreated and prebiotic-treated transgenic rats. Total cecal SCFA concentrations were 92 ± 17.3 (untreated) and 102 ± 37.7 (prebiotics) µmol/g, and consisted primarily of acetate (76 ± 4.9 untreated versus 82 ± 7.6% prebiotics), propionate (15 ± 3.6 untreated versus 11 ± 5.8% prebiotics), and butyrate (7 ± 1.1 untreated versus 8 ± 3.1% prebiotics) (data not shown).

Discussion

Members of the gut microbiota do not induce disease in the normal host, whereas chronic intestinal inflammation can develop in the genetically susceptible host in response to the same intestinal bacteria.[3] Manipulating the gut microbiota might be of importance in order to change the natural course of colitis. For example, in several models of experimental colitis, antibiotic treatment decreased the amounts of intestinal disease-inducing bacteria and prevented inflammation,[6,25,32] whereas increased amounts or the addition of certain strains of colonic probiotic bacteria could prevent disease.[10,11,33]

We showed in the present study that a combination of inulin and oligofructose partially prevented intestinal inflammation in specific pathogen-free HLA-B27 transgenic rats. This is the first study in which prebiotics were used as primary therapy in genetically susceptible rodents. The results are in agreement with findings by Schultz et al in a recent study, which reported that a probiotic preparation that also contained inulin attenuated colitis development HLA-B27 rats.[34] Our results also confirmed previous studies in induced models of colitis, in which prebiotic treatment resulted in attenuation of colitis. In rats, oral inulin decreased distal colonic lesions in

DSS-induced colitis,[17] and oral fructo-oligosaccharides decreased TNBS-induced colitis, although its effects were apparent only after 14 days of feeding.[18] No effects were seen after oral administration of trans-galacto-oligosaccharide started before or during TNBS-colitis induction,[35] indicating that not all prebiotic preparations are equally effective in preventing disease. In patients with mild to moderate ulcerative colitis, prebiotics such as germinated barley food extracts decreased clinical and endoscopic evidence of inflammation in both a small placebo-controlled trial as well as in an open-label study.[36,37] Inulin administration to ulcerative colitis patients with pouchitis after colectomy led to a reduction of inflammation in the pouch mucosa.[38]

Changes in the gut microbiota composition, especially growth promotion of beneficial bacteria induced by prebiotics, may contribute to colitis prevention.[16] In this study we analyzed intestinal microbiota changes using molecular techniques such as PCR-DGGE and FISH. Analysis of the cecal and colonic microbiota using PCR-DGGE showed alterations to the microbiota profile induced by prebiotic treatment in HLA-B27 transgenic rats. Prebiotic administration to transgenic rats resulted in the detection of a 16S rRNA gene fragment that was absent in untreated transgenic rats. Sequence analysis revealed that this band represented *Bifidobacterium animalis*. A similar microbiotic spectrum in cecal contents of transgenic rats was found by PCR-DGGE after feeding probiotics plus inulin.[34] Interestingly, in the latter study added probiotic bacteria could not be detected, indicating that prebiotic inulin mediated the changes in cecal microflora. Quantitative analysis of the cecal and colonic content by FISH in our study confirmed that the prebiotic combination significantly increased cecal and colonic *Bifidobacteria*, but it also showed an increased amount of intestinal *Lactobacilli*, as shown in table 1. Increasing concentrations of intestinal probiotic bacteria by oral administration has been effective in experimental colitis as well as human IBD.[11,13,33] Oral administration of *Lactobacilli* and *Bifidobacteria* decreased colitis in TNBS-induced colitis and caused similar changes in cecal microbiota composition as those induced by fructo-oligosaccharides. Adding *Lactobacilli* and *Bifidobacteria* to topical butyrate solutions in rats with TNBS-induced colitis was necessary to induce a similar disease improvement as shown by oral fructo-oligosaccharides administration, suggesting that the increase of protective bacteria mediated the clinical efficacy of prebiotics.[18] Feeding germinated barley foodstuff to humans also stimulated growth of *Bifidobacteria* as well as *Eubacterium* species and increased the production of butyrate and other SCFA.[39] Thus, increased probiotic intestinal bacteria are associated with prevention of colitis in the present study, and might (partially) mediate the beneficial effects of prebiotic administration.

B. vulgatus and possibly other anaerobic bacteria play a pathogenic role in colitis development in transgenic rats and in human IBD. This was demonstrated by the induction of colitis in transgenic rats.[8,20] Moreover, metronidazole attenuated colitis in this model and is an effective adjunct therapy in Crohn's colitis.[6,32] Probiotic bacteria are reported to suppress the growth of pathogenic intestinal bacteria. For example, *Bifidobacterium infantis* suppressed growth of *B. vulgatus*.[40] Similarly, *Bifidobacteria*-fermented milk decreased fecal *B. vulgatus* concentrations in ulcerative colitis.[41] Inulin and oligofructose also reduced luminal *Clostridium difficile* populations in healthy volunteers, which was associated with a bifidogenic effect.[42] Despite a trend towards a decreased intestinal population of *Bacteroides*/*Prevotella* relative to the total amount of luminal bacteria after feeding inulin/oligofructose in transgenic rats, this trend did not reach statistical significance. We also did not observe any significant differences in the amounts of cecal *Clostridia*-group XIVa between the experimental groups. Taken together, this would suggest that prebiotics and/or probiotic *Bifidobacteria* and *Lactobacilli* did not mediate protection by suppressing the growth of *Bacteroides* species in transgenic rats.

Prebiotics that escape digestion in the upper intestine are important sources of energy for cecocolonic bacteria. The end-products of prebiotic fermentation by these organisms lead to the production of lactate and SCFA, such as acetate, propionate and butyrate. Changes in SCFA production, especially increased luminal butyrate concentrations, can also contribute to down-regulation of proinflammatory cascades in models of experimental colitis and human IBD.[17,36,39,43-47] However, we did not detect significant differences in luminal SCFA concentrations in prebiotic- versus untreated transgenic rats, indicating that prebiotics did not mediate protection by changes in luminal SCFA concentrations.

A widely accepted hypothesis for the pathogenesis of chronic intestinal inflammation, such as IBD, is an overly aggressive T_H1 mediated cytokine response to commensal bacteria. We recently showed that cecal bacteria induce a proinflammatory cytokine response in colitis-susceptible transgenic rats, whereas the same bacteria induce anti-inflammatory cytokines, such as an increase of IL-10 and TGF-β, in mesenteric lymph nodes and cecal mucosa of disease-tolerant non-transgenic littermates.[11,19] In our current study we report that cecal bacterial lysate-stimulated mesenteric lymph node cells derived from prebiotic-treated transgenic rats produced less IFN-γ than those from untreated transgenic rats. Prebiotic treatment also significantly decreased cecal IL-1β production, which correlated with less cecal and colonic inflammation. Decreased production of mucosal proinflammatory cytokines is in agreement with probiotic treatment studies in experimental colitis[10,11,33] and refractory pouchitis after colectomy for ulcerative colitis.[13]

Of major interest, we report for the first time that prebiotic treatment of transgenic rats significantly increased cecal TGF-β. This finding indicates that prebiotics can exert immunomodulatory effects, either directly or secondarily through increase of intestinal probiotic bacteria. Indeed, probiotics administered to pregnant and lactating mothers increased the amount of TGF-β in the breast milk.[48] The importance of TGF-β in immune homeostasis is demonstrated by the fact that TGF-β deficient mice die within five weeks of severe multiple organ inflammation.[49] TGF-β is also important for the regulation of intestinal inflammation. Colitis develops in SCID mice restored with a mixture of $CD45RB^{hi}$ CD4 and $CD45RB^{low}$ CD4 cells that were treated with anti-TGF-β mAb.[50] Furthermore, intranasal administration of a TGF-β-containing plasmid prevented TNBS induced colitis.[51] Therefore, increased cecal TGF-β could mediate some of the beneficial effects of prebiotics.

In conclusion, we showed in HLA-B27 transgenic rats that oral administration of the prebiotic combination inulin and oligofructose partially prevents colitis. Protection induced by these prebiotics correlated not only with changes in intestinal microbiota towards an increase of luminal *Bifidobacteria* and *Lactobacilli*, but was also associated with immunomodulatory effects. Although prebiotics can mediate protective effects through *Bifidobacteria* and *Lactobacilli*, it is also possible that prebiotics are beneficial by direct immunosuppressive or immunomodulatory effects on the host. These studies suggest that prebiotics may have potential as adjuvant maintenance therapy for the treatment of human IBD.

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II

Bacterial-Induced Immune Responses in Experimental Colitis

Reduced ratio of protective versus proinflammatory cytokine responses to commensal bacteria in HLA-B27 transgenic rats

6

†Levinus A Dieleman[1], †Frank Hoentjen[1,2], Bi-Feng Qian[1]
Dave Sprengers[1], Eric Tjwa[1], Marcia F. Torres[1]
Chad D. Torrice[1], R. Balfour Sartor[1], and Susan L. Tonkonogy[3]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

[3]College of Veterinary Medicine
North Carolina State University, Raleigh, USA

†contributed equally to this manuscript

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Abstract

Introduction and aims

Germ-free HLA-B27 transgenic rats do not develop colitis, but colonization with specific pathogen-free bacteria induces colitis accompanied by immune activation. To study host-dependent immune responses to commensal cecal bacteria we investigated cytokine profiles in mesenteric lymph node cells from HLA-B27 transgenic versus non-transgenic littermates after *in vitro* stimulation with cecal bacterial lysates.

Methods

Supernatants from cecal bacterial lysate-stimulated unseparated and T or B cell-depleted mesenteric lymph node cells from HLA-B27 transgenic and non-transgenic littermates were analyzed for IFN-γ, IL-12, TNF, IL-10 and TGF-β production.

Results

Our results show that unfractionated transgenic mesenteric lymph node cells stimulated with cecal bacterial lysate produced more IFN-γ, IL-12, and TNF than did non-transgenic mesenteric lymph node cells. In contrast, cecal bacterial lysate-stimulated non-transgenic mesenteric lymph node cells produced more IL-10 and TGF-β. T cell depletion abolished IFN-γ and decreased IL-12 production, but did not affect IL-10 and TGF-β production. Conversely, neither IL-10 nor TGF-β was produced in cultures of B cell-depleted mesenteric lymph nodes. In addition, $CD4^+$ T cells enriched from mesenteric lymph nodes of HLA-B27 transgenic but not from non-transgenic rats produced IFN-γ when co-cultured with cecal bacterial lysate-pulsed antigen presenting cells from non-transgenic rats. Interestingly, IL-10 and TGF-β, but not IFN-γ, IL-12, and TNF were produced by mesenteric lymph node cells from germ-free transgenic rats.

Conclusions

These results indicate that the colitis that develops in specific pathogen-free HLA-B27 transgenic rats is accompanied by activation of IFN-γ-producing $CD4^+$ T cells that respond to commensal bacteria. However, B cell cytokine production in response to components of commensal intestinal microorganisms occurs in the absence of intestinal inflammation.

Introduction

In recent years several studies have emphasized the role of commensal intestinal bacteria in the pathogenesis of experimental chronic immune-mediated intestinal inflammation and human IBD. This is most clearly demonstrated in a wide variety of genetically engineered and induced rodent models in which the susceptible host develops spontaneous colitis in the presence of non-pathogenic resident intestinal organisms.[1] In most rodent models chronic intestinal inflammation is mediated by the T_H1 cytokines IL-12 and IFN-γ, while suppression of inflammation is mediated by the regulatory cytokines IL-10 and TGF-β. The influence of resident flora on the initiation and perpetuation of spontaneous colitis, gastritis and arthritis has been well characterized in HLA-B27/β2 microglobulin transgenic rats, which develop disease by three months of age when raised under specific pathogen-free conditions.[2] When raised in a germ-free environment these rats fail to develop gastritis, colitis and arthritis.[3,4] However, these animals develop colitis and gastritis within one month after transfer to a specific pathogen-free environment.[3] In this model *B. vulgatus* preferentially induces colitis after monoassociation for one month, with no inflammation resulting from *E. coli* monoassociation.[5] The importance of host genetic susceptibility was illustrated by the lack of colitis or activation of immune responses in wild-type non-transgenic littermates colonized with the same specific pathogen-free bacteria or *B. vulgatus*.[3,5] The role of resident intestinal flora in the pathogenesis of colitis in transgenic rats is further emphasized by an increase of cecal inflammation after the creation of a blind cecal loop which results in an increased bacterial load, including *Bacteroides* species,[6] and studies showing that broad spectrum antibiotics can prevent as well as treat colitis.[7] In other experimental models of colitis, similar findings have been reported.[8-9]

Although these observations suggest a central role for normal luminal bacteria in the induction and perpetuation of immune-mediated colitis in this model, the mechanisms by which bacteria activate immune cells responsible for the development of colitis remain unclear. The aim of our study was to investigate cytokine responses induced in HLA-B27 transgenic rats and their non-transgenic littermates by commensal enteric bacteria and their products. We show that lysates of cecal contents induce mesenteric lymph node cells to produce an array of cytokines. Mesenteric lymph node $CD4^+$ cells from transgenic rats, but not from non-transgenic rats, produce IFN-γ, which is dependent on *in vivo* bacterial colonization. Both transgenic and non-transgenic mesenteric lymph node B cells produce IL-10 and TGF-β. The production of these two regulatory cytokines is independent of prior bacterial colonization.

Materials and methods

Animals

HLA-B27 transgenic rats (the 33-3 line on the F344 background) and their non-transgenic littermates, originally obtained from Dr. Joel D. Taurog, University of Texas Southwestern Medical Center, were maintained in specific pathogen-free housing conditions at the University of North Carolina, Chapel Hill or in germ-free conditions in the Gnotobiotic Animal Core of the Center for Gastrointestinal Biology and Disease at the College of Veterinary Medicine, NC State University in Raleigh, NC. Presence or absence of the HLA-B27 transgene was determined by PCR using DNA isolated from tail clippings. Rats between the ages of four and six months were used for our studies. All studies were approved by University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Histology

Colons and ceca were fixed and stained as previously described.[3] A validated histologic inflammatory score ranging from 0 to 4 was used for blinded evaluation.[3]

Preparation of mesenteric lymph node cells and enrichment of lymphoid cell subpopulations

Mesenteric lymph nodes were removed from HLA-B27 transgenic and non-transgenic rats, and single cell suspensions were prepared by gentle teasing. Magnetic bead separation, using antibody coated microbeads and columns designed for cell depletion (Miltenyi Biotec, Auburn, CA) was performed according to the manufacturer's instructions using the following reagents: LD columns; Rat Pan T Cell Microbeads, clone OX-52, for T cell depletion; Rat CD45RA, clone OX-33, for B cell depletion; Rat CD4 Microbeads, clone OX-38, for $CD4^+$ cell depletion; Rat CD8a Microbeads, clone G38, for $CD8^+$ cell depletion; and Rat CD45RA plus Rat CD8a Microbeads to enrich $CD4^+$ cells.

Preparation of cecal bacterial lysates

Cecal bacterial lysates were prepared as described by Cong et al.[10] Briefly, cecal contents from several non-transgenic or transgenic rats were solubilized by vortexing in RPMI, and incubated with 10 μg/ml DNA-ase, 0.01 M magnesium chloride and then homogenized for 3 minutes using 0.1 mm glass beads in a Mini-Bead Beater (Biospec Products, Bartlesville, OK). After centrifuging at 10,000*g* for 10 min the supernatant was filtered through a 0.45 μm syringe filter. Sterility was confirmed by aerobic and anaerobic culture.

Mesenteric lymph node cell cultures

Either unseparated mesenteric lymph node cells, or cell subpopulations obtained after antibody-coated magnetic bead depletion, were washed and $4x10^5$ cells were cultured in 96 well flat bottom microplates (Costar 3595), in 0.2 ml complete medium (RPMI 1640 plus 5% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, $5x10^{-5}$ M 2-mecaptoethanol, and 50 μg/ml gentamicin) for three days, which we found to be optimal for detection of all of the cytokines measured with the exception of TGF-β. Similar relative differences in cytokines produced by transgenic versus non-transgenic mesenteric lymph node cells were present at each time point between 12 hours and six days. For analysis of TGF-β production, $8x10^5$ cells were cultured for five days in serum-free RPMI which was supplemented with 1% Nutridoma-SP (Roche Molecular Biochemicals, Mannheim, Germany). Higher levels of TGF-β were consistently found in supernatants of day five compared to day three cultures. Cells were stimulated with different concentrations of cecal bacterial lysate as indicated for each experiment. Culture supernatants were collected and stored at –20°C.

Preparation of antigen-presenting cells and co-cultures with $CD4^+$ cells

We used T-cell depleted mesenteric lymph node cells from non-transgenic rats for antigen-presenting cells. T cells were lysed using IgM anti-rat CD3 (clone 1F4, Pharmingen, San Diego, CA), followed by incubation with rabbit complement (normal rabbit serum prepared in this laboratory). The cells remaining after complement-mediated lysis of $CD3^+$ cells were >95% surface Ig positive, <2% CD4 positive and <2% CD8 positive. The cells were pulsed overnight with cecal bacterial lysate from non-transgenic rats (100 μg/ml) or with unrelated protein antigen keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) in complete medium. The pulsed

antigen-presenting cells were then washed to remove excess antigens and other bacterial products. Magnetic bead-enriched $CD4^+$ cells, $2x10^5$ per well, were stimulated with antigen-pulsed antigen-presenting cells, $3x10^5$ per well. Supernatants were harvested after 3 days and stored at -20°C.

Flow cytometry

Mesenteric lymph node cells before and after magnetic bead separation were evaluated by flow cytometry using the following fluorochrome labeled or unlabeled reagents. For detection of HLA-B27-expressing cells, we used culture supernatant from the murine hybridoma, designated ME-1, obtained from ATCC (Rockville, MD), followed by FITC labeled goat anti-mouse IgG (γ chain specific) antibody (Southern Biotechnology, Birmingham, AL). For surface immunoglobulin positive B cells, we used FITC labeled goat anti-rat IgG (H + L) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). For $CD4^+$ and $CD8^+$ cells we used PE-anti-CD4 monoclonal antibody (clone W3/25) and FITC anti-CD8 monoclonal antibody (clone OX-8), (Caltag, Burlingame, CA) respectively.

Cytokine and PGE_2 measurements

Cytokines in cell culture supernatants were measured by ELISA using unlabeled capture antibodies and biotin-labeled detection antibodies, followed by horse-radish peroxidase labeled Streptavidin. The concentration of each cytokine was determined by comparison to a standard curve generated using recombinant proteins. For IFN-γ, we used unlabeled polyclonal anti-IFN-γ antibody and biotin-labeled monoclonal anti-IFN-γ antibody (clone DB-1) (Biosource International, Camarillo, CA). For IL-10 we used unlabeled monoclonal anti-rat IL-10 antibody (clone A5-7) and biotin-labeled monoclonal anti-rat IL-10 antibody (clone A5-6) (BD Biosciences Pharmingen, San Diego, CA). For IL-12 we used a rat IL-12 p40 CytoSet (Biosource International). For TNF we used a rat TNF BD OptEIA ELISA Set (BD Biosciences Pharmingen). TGF-β concentrations were measured after acidification and neutralization according to the manufacturer using a TGF-β1 specific ELISA (Promega, Madison, WI). PGE_2 was measured using a competitive immunoassay Correlate-EIA (Assay Designs Inc., Ann Arbor MI).

Cytokine mRNA expression in cecal tissues

Total RNA was extracted from cecal tissue using a standard technique as described previously.[11] One microgram of RNA isolated from each sample was reverse transcribed, and the cDNA (1 - 2 μg) was then amplified using primers specific for rat cytokines.[11-16] Negative controls without cDNA were included in each experiment. Aliquots of all samples were analyzed by electrophoresis on 2% agarose gel containing GelStar (BioWhittaker). The size of the PCR product was compared to the predicted size using a 100 bp DNA ladder (Gibco BRL, Grand Island, NY). The DNA products were visualized by ultraviolet fluorescence and photographed (Polaroid 665, Polaroid Corp, Cambridge, MA). The cytokine mRNA was quantified by densitometry, and the ratio to β-actin mRNA was calculated.

Statistical analysis

Cytokine levels are expressed as mean ± standard deviation of triplicate measurements. A non-paired Student t-test or alternate Welch t-test was used, in which a two-tailed p value of < 0.05 was considered statistically significant.

Results

Evaluation of colitis and of lymphoid cell subpopulations in mesenteric lymph nodes of specific pathogen-free and of germ-free transgenic and non-transgenic rats

Transgenic rats housed in specific pathogen-free conditions showed significant gross and histologic evidence of colitis of the entire colon and cecum, with over 95% of the transgenic rats demonstrating histology scores higher than 3.0 on a 0-4 scale. In contrast, their non-transgenic littermates exposed to the same microflora did not develop colitis, nor did germ-free transgenic or non-transgenic rats exhibit any disease (figure 1).

Specific pathogen-free transgenic rats with colitis had larger mesenteric lymph nodes, containing over 3-fold more cells, than did mesenteric lymph nodes of their non-transgenic littermates (table 1). Mesenteric lymph nodes from specific pathogen-free transgenic rats contained a significantly higher percentage and total number of $CD4^+$ T cells than non-transgenic mesenteric lymph node (table 1). The percentage of $CD8^+$ T cells and of B cells was lower in transgenic versus non-transgenic mesenteric lymph nodes. However, due to the higher number of total mesenteric lymph node cells, transgenic mesenteric lymph nodes contained significantly more $CD8^+$ cells and B cells than did mesenteric lymph nodes of non-transgenic littermates.

Mesenteric lymph nodes of germ-free rats were small and contained much lower cell numbers than mesenteric lymph nodes of specific pathogen-free rats. The total number of mesenteric lymph node cells was not significantly different between germ-free transgenic and germ-free non-transgenic rats. Mesenteric lymph nodes from germ-free transgenic rats contained higher proportions and higher total numbers of $CD4^+$ cells but lower proportions and fewer total numbers of B cells compared to mesenteric lymph nodes of non-transgenic littermates, whereas the percentages and numbers of $CD8^+$ cells were similar (table 1). All animals identified as HLA-B27 transgenic by PCR analysis of tail DNA also expressed HLA-B27 on mesenteric lymph node cells as determined by flow cytometry.

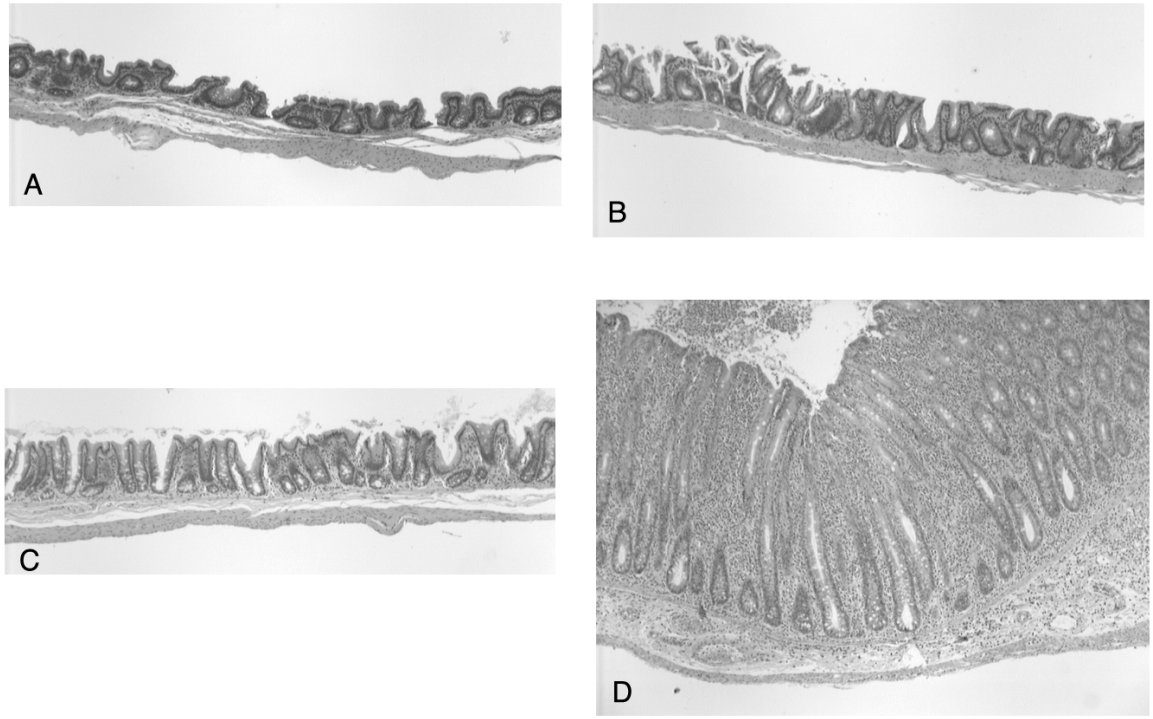

Figure 1. Representative photomicrographs of tissue sections (x40) from ceca of five to six month old (A) germ-free non-transgenic; (B) germ-free transgenic; (C) specific pathogen-free non-transgenic; (D) specific pathogen-free transgenic rats. Note the extensive mucosal and submucosal inflammation as well as significant crypt hyperplasia in the cecum from a specific pathogen-free transgenic rat (D), whereas no colitis was present in cecal tissue from germ-free rats or in the cecum from non-transgenic rats housed in an specific pathogen-free environment (A-C).

	Specific Pathogen-Free		Germ-Free	
	Non-Transgenic n=15	Transgenic n=15	Non-Transgenic n=16	Transgenic n=12
Total Cells (x 10^6)	87.6 ± 6.2	279.8 ± 22.7 [a]	41.8 ± 4.8	41.7 ± 5.5
Percent				
$CD4^+$	40.3 ± 0.9	56.2 ± 1.1[a]	28.1 ± 1.4	42.2 ± 1.9[a]
$CD8^+$	16.7 ± 0.6	13.4 ± 0.5[c]	11.9 ± 0.8	13.5 ± 0.6
Surface Ig	36.4 ± 1.8	24.9 ± 1.2[a]	51.3 ± 2.7	38.7 ± 2.1[b]
Cells x 10^6				
$CD4^+$	35.5 ± 2.9	155.6 ± 11.9[a]	11.4 ± 1.1	17.7 ± 2.7[b]
$CD8^+$	14.7 ± 1.2	37.6 ± 3.5[a]	5.0 ± 0.7	5.8 ± 1.0
Surface Ig	31.9 ± 2.7	70.3 ± 6.8[a]	22.2 ± 3.4	16.0 ± 2.1

Table 1. Cell numbers and cell subpopulations in mesenteric lymph nodes of specific pathogen-free and germ-free HLA-B27 transgenic rats and their non-transgenic littermates. Values represent mean ± SEM of cell numbers or of percentages of the different lymphoid cell subpopulations. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ versus non-transgenic littermates.

Cytokine production in unseparated mesenteric lymph node cells from specific pathogen-free transgenic versus non-transgenic rats

Unseparated transgenic mesenteric lymph node cells, stimulated *in vitro* with 100 μg/ml cecal bacterial lysate produced significantly more IFN-γ, IL-12, and TNF compared to mesenteric lymph node cells from non-transgenic rats (figures 2A, B, and C). In contrast, non-transgenic mesenteric lymph node cells stimulated with 10 μg/ml cecal bacterial lysate produced significantly more IL-10 than transgenic mesenteric lymph node cells (figure 2D). TGF-β production by mesenteric lymph node cells followed the same trend as IL-10 after 10 μg/ml and also 100 μg/ml cecal bacterial lysate stimulation; non-transgenic unseparated mesenteric lymph node cells produced significantly more TGF-β than those from transgenic littermates (figure 2E). PGE_2 concentrations did not differ significantly in cecal bacterial lysate stimulated mesenteric lymph node cell cultures from transgenic versus non-transgenic rats (data not shown).

Stimulation with cecal bacterial lysate obtained from either transgenic rats or from non-transgenic rats showed the same pattern; both lysates induce more IFN-γ, IL-12, and TNF, and less IL-10 and TGF-β in mesenteric lymph node cell cultures of transgenic compared to non-transgenic rats (data not shown). In five separate experiments, we did not observe consistent differences in cytokine levels induced by bacterial lysates from either transgenic or from non-transgenic rats. Therefore, we chose to use cecal bacterial lysate from non-transgenic rats for the remainder of our experiments.

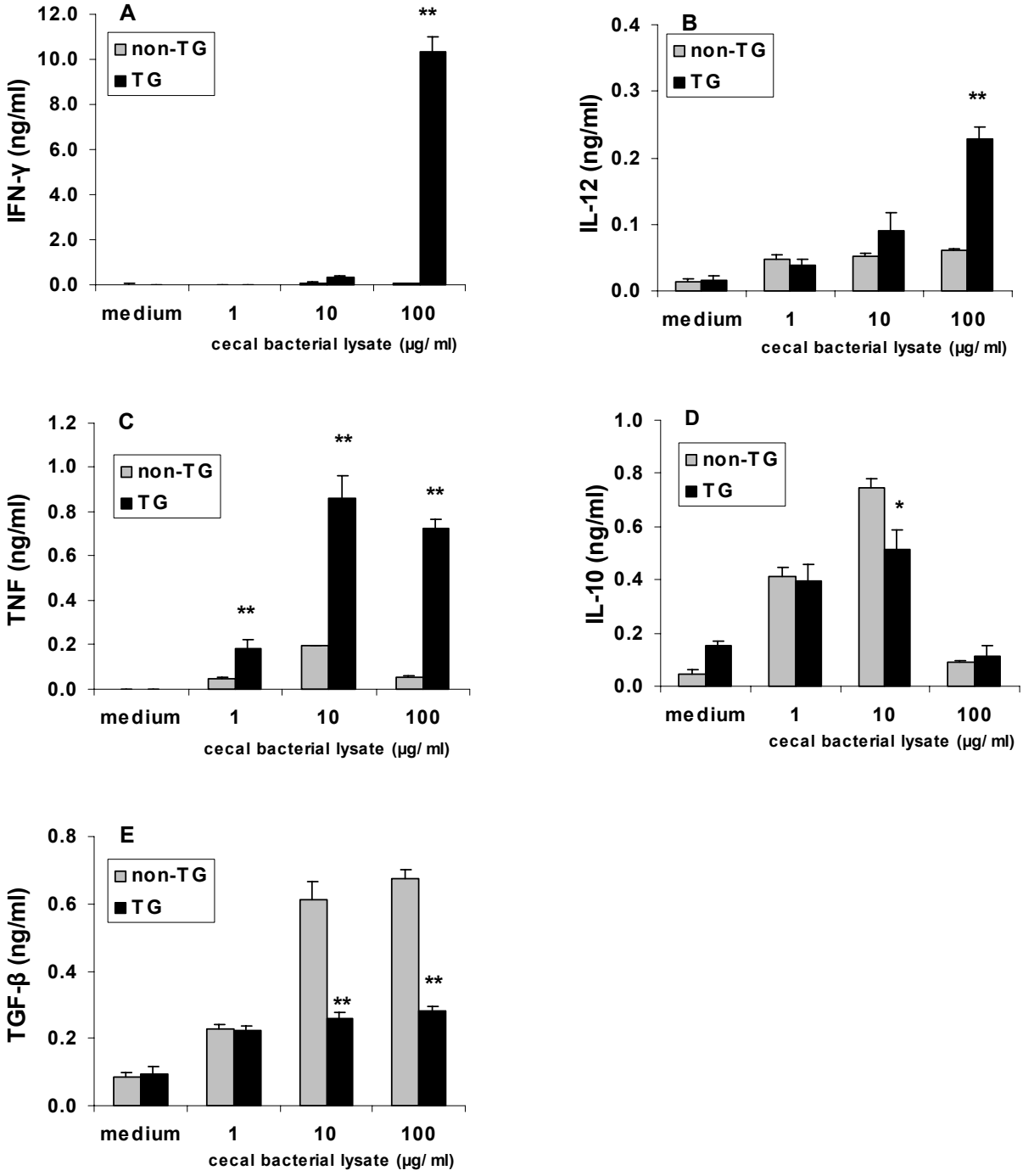

Figure 2. Cytokine levels in supernatants of unseparated mesenteric lymph node cell cultures from five month old specific pathogen-free non-transgenic or transgenic rats after stimulation with various concentrations of cecal bacterial lysate (in µg/ml) from non-transgenic rats. (A) IFN-γ; (B) IL-12 (p40); (C) TNF; (D) IL-10; (E) TGF-β. Data shown are from a representative experiment out of two to seven separate experiments. Cytokines levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants. ** $p < 0.01$ versus non-transgenic mesenteric lymph node cell supernatants, * $p < 0.05$ versus non-transgenic mesenteric lymph node cell supernatants. Non-TG, non-transgenic; TG, transgenic.

Cecal cytokine mRNA expression in specific pathogen-free transgenic versus non-transgenic rats

The mRNA expression of proinflammatory cytokines was significantly higher in cecal tissues from transgenic compared to non-transgenic rats (figure 3). The cytokine/β-actin ratios for IL-1β, IFN-γ as well as for IL-12 were significantly higher in transgenic rats compared to non-transgenic littermates. Consistent with results from cecal bacterial lysate-stimulated unseparated mesenteric lymph node cell cultures, cecal IL-10 mRNA expression was significantly higher in non-transgenic versus transgenic rats. However, cecal mRNA expression for TGF-β did not differ between transgenic and non-transgenic rats.

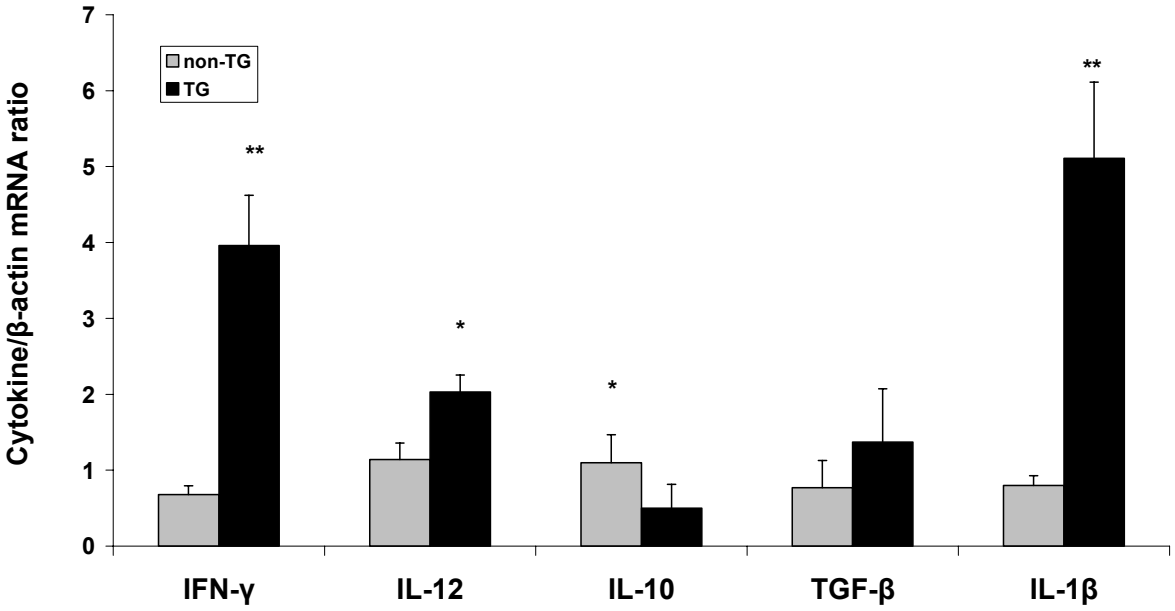

Figure 3. Expression of IFN-γ, IL-12, IL-10, TGF-β and IL-1β mRNA in cecal tissues from specific pathogen-free transgenic rats and their non-transgenic littermates. Total RNA was reverse transcribed and amplified for various cycles by the polymerase chain reaction. Cytokine and β-actin mRNA expression was evaluated in eight non-transgenic and six transgenic rats. Tissue from each individual animal was analyzed separately. The results shown represent the averages of the ratios of specific cytokine versus β-actin mRNA obtained in two to three separate experiments.
** $p < 0.01$ versus non-transgenic cecal tissue, * $p < 0.05$ versus non-transgenic cecal tissue.
Non-TG, non-transgenic; TG, transgenic.

Cytokine production in mesenteric lymph node cells from germ-free transgenic versus non-transgenic rats

Cecal bacterial lysate stimulation of mesenteric lymph node cells from germ-free rats did not induce IFN-γ, IL-12 or TNF production compared to unstimulated levels (data not shown), indicating that production of these cytokines in transgenic rats depends on *in vivo* exposure to commensal bacteria and their products. In contrast, mesenteric lymph node cells from both transgenic and non-transgenic germ-free rats produced IL-10 and TGF-β after stimulation with bacterial lysate (figure 4), indicating that these two cytokines can be produced by naive cells independent of *in vivo* exposure to commensal intestinal microorganisms.

As in specific pathogen-free rats, IL-10 detected in the supernatants of mesenteric lymph node cell cultures from germ-free non-transgenic rats was significantly more than that from germ-free transgenic rats after stimulation with 1 μg/ml and with 10 μg/ml cecal lysate (figure 4A). After stimulation with the optimal dose of 100 μg/ml, germ-free non-transgenic mesenteric lymph

node cells produced almost twice as much TGF-β compared to germ-free transgenic mesenteric lymph node cells (figure 4B).

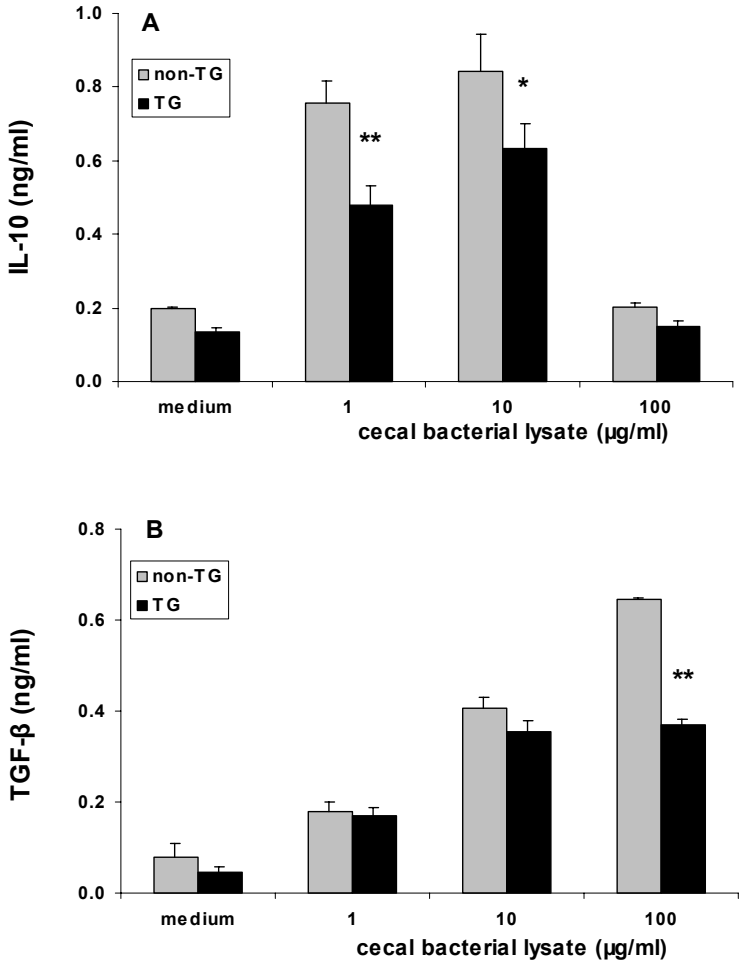

Figure 4. Cytokine levels in supernatants of unseparated mesenteric lymph node cell cultures from four to five month old germ-free non-transgenic or transgenic rats after stimulation with various concentrations of cecal bacterial lysates (in µg/ml) from non-transgenic rats. (A) IL-10; (B) TGF-β. Data shown are from a representative experiment out of five (IL-10) or two (TGF-β) separate experiments. Cytokines levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants. ** $p < 0.01$ versus non-transgenic mesenteric lymph node cell supernatants, * $p < 0.05$ versus non-transgenic mesenteric lymph node cell supernatants.

Cytokine production in mesenteric lymph node cells after T cell or B cell depletion or in co-cultures of CD4 cells plus antigen-pulsed antigen-presenting cells

To determine which cell type(s) are critical to the production of the cytokines that we evaluated, we carried out negative selection to deplete T cells or B cells from mesenteric lymph node cell preparations. After T cell depletion less than 2.5% of the total remaining cells were CD4+/CD8+, as determined by flow cytometry. Approximately 97% of the T cell-depleted mesenteric lymph node cells were positive for surface Ig, which is characteristic of B cells. In response to 100 µg/ml of cecal bacterial lysate, T cell-depleted mesenteric lymph node cells from transgenic rats did not produce IFN-γ (figure 5A). IFN-γ production was dramatically reduced but not absent in cultures of B cell-depleted mesenteric lymph node cells. CD4+ cell depletion of

mesenteric lymph node cells, with less than 0.5 % remaining CD4+ cells, significantly reduced IFN-γ production to the same degree as T cell depletion (figure 5B). In contrast, CD8+ cell depletion of mesenteric lymph node cells, with less than 0.5% CD8+ cells, had no effect on IFN-γ production after bacterial lysate stimulation (figure 5B).

IL-12 production was significantly reduced after T cell depletion in transgenic mesenteric lymph node cells in response to 100 μg/ml cecal bacterial lysate (figure 5C). Supernatants of B cell-depleted mesenteric lymph node cell cultures contained slightly more IL-12 than did supernatants of unseparated mesenteric lymph node cell cultures.

To more precisely evaluate $CD4^+$ T cell responses to cecal bacterial lysate, we co-cultured CD4-enriched mesenteric lymph node cells with cecal bacterial lysate pulsed antigen-presenting cells. As shown in figure 6, $CD4^+$ cells from transgenic but not from non-transgenic mesenteric lymph nodes produced high amounts of IFN-γ in co-culture with cecal bacterial lysate pulsed antigen-presenting cells. Interestingly, the *in vitro* response is not dependent on antigen presentation via the HLA-B27 molecule because $CD4^+$ cells from transgenic rats respond to antigen-pulsed non-transgenic antigen-presenting cells. Further studies focusing on the potential differences between antigen-presenting cells from transgenic and from non-transgenic rats to present bacterial antigens are in progress.

In contrast to IFN-γ and IL-12, supernatants of cecal bacterial lysate stimulated T cell-depleted mesenteric lymph node cells contained equivalent or greater amounts of IL-10 and TGF-β compared to supernatants of unseparated mesenteric lymph node cells, indicating that these two cytokines are not produced by T cells (figure 7). Moreover, the levels of IL-10 and TGF-β in supernatants of B cell depleted mesenteric lymph node were even lower than amounts in supernatants of unstimulated cells, indicating that B cells either produce the majority of the IL-10 and TGF-β or that B cells are essential to their production (figure 7).

As also shown in figure 7, IL-10 and TGF-β levels were lower in supernatants of T cell-depleted transgenic compared to non-transgenic mesenteric lymph node cells after cecal bacterial lysate stimulation. In a total of seven separate experiments, there is a trend towards production of lower amounts of IL-10 by T cell-depleted transgenic mesenteric lymph node cells compared to non-transgenic cells (ratio IL-10 transgenic:IL-10 non-transgenic = 0.78 ± 0.06). These results suggest that there is only a marginal difference between the abilities of transgenic and non-transgenic mesenteric lymph node cells to produce IL-10 in response to commensal bacteria and their products.

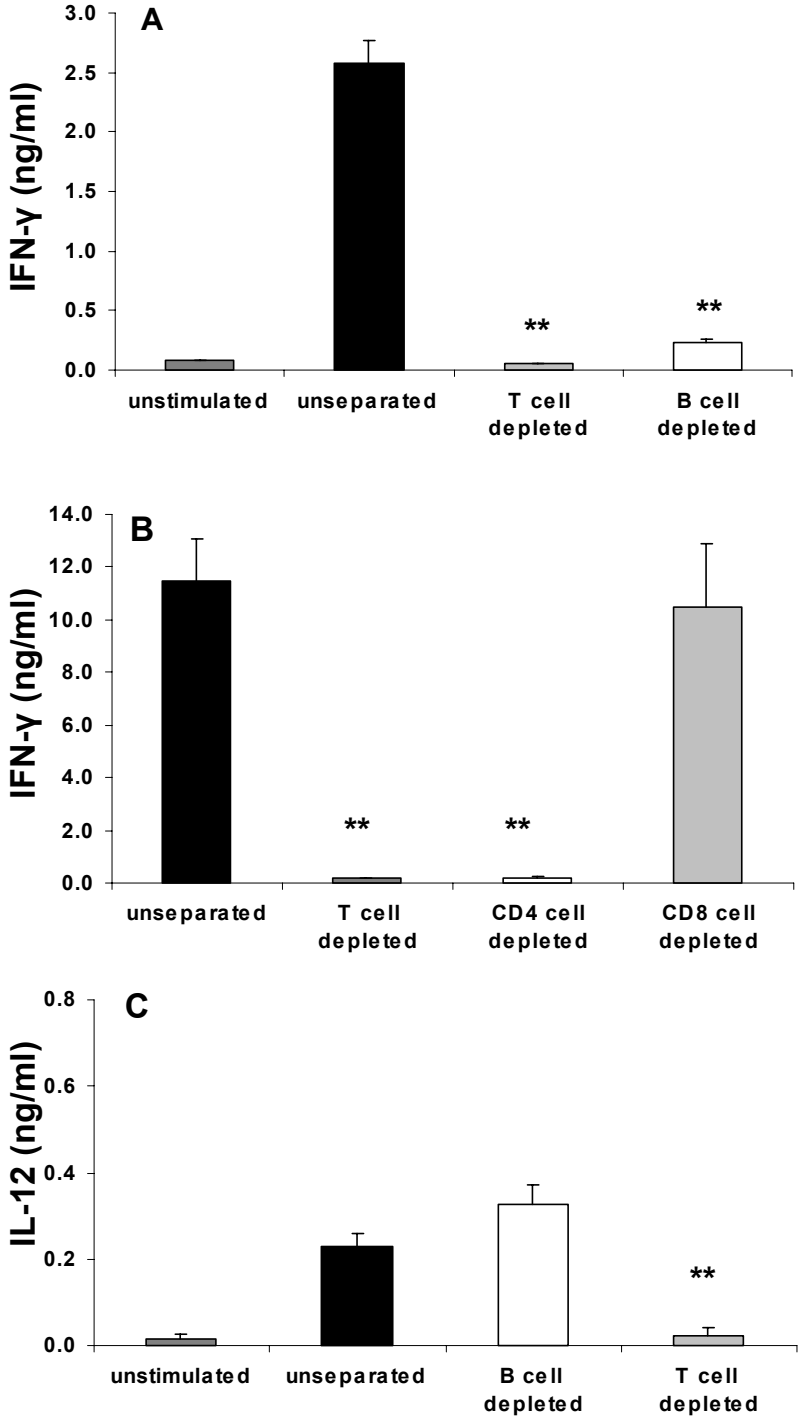

Figure 5. Cytokine levels in mesenteric lymph node cell cultures from four to five month old specific pathogen-free transgenic rats. (A, B) IFN-γ and (C) IL-12 production by transgenic mesenteric lymph node cells which were either unseparated, T cell depleted, $CD4^+$ cell depleted, $CD8^+$ cell depleted or B cell depleted, then stimulated with 100 μg/ml cecal bacterial lysate from non-transgenic rats. Cytokine levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants and are representative of three separate experiments. **$p < 0.01$ versus unseparated mesenteric lymph node cell supernatants.

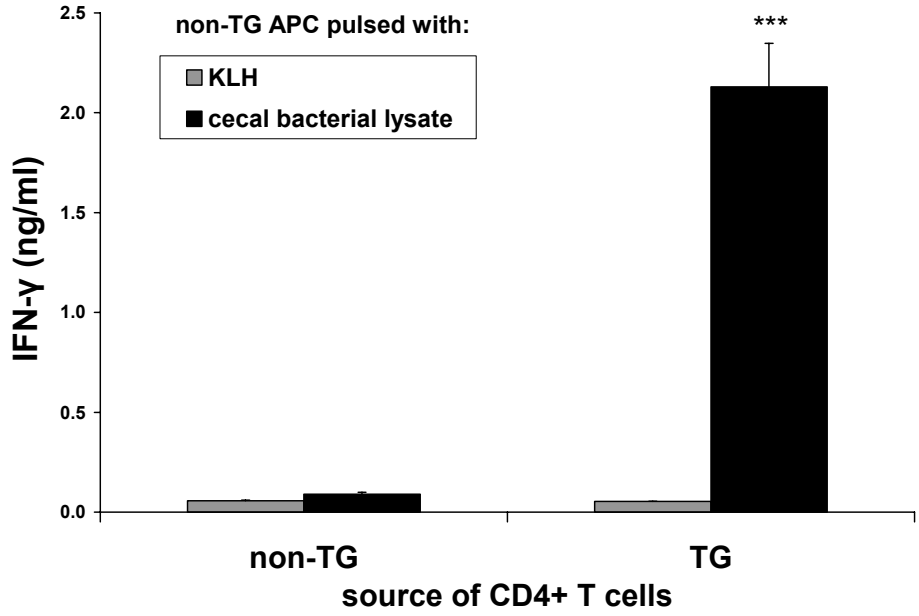

Figure 6. IFN-γ production by $CD4^+$ mesenteric lymph node T cells in co-culture with cecal bacterial lysate-pulsed antigen-presenting cells. $CD4^+$ T cells were enriched from mesenteric lymph nodes of specific pathogen-free transgenic or non-transgenic rats and stimulated for three days with either cecal bacterial lysate-pulsed or KLH-pulsed antigen-presenting cells prepared from mesenteric lymph nodes of non-transgenic rats as described in Materials and Methods. Cecal bacterial lysate-pulsed antigen-presenting cells alone do not produce detectable amounts of IFN-γ. Values represent the mean and standard deviation of IFN-γ in triplicate culture supernatants and are representative of six separate experiments. ***$p<0.005$ versus IFN-γ in supernatants of transgenic $CD4^+$ mesenteric lymph node cells in co-culture with KLH-pulsed antigen-presenting cells and versus IFN-γ in supernatants of non-transgenic $CD4^+$ mesenteric lymph node cells in co-culture with cecal bacterial lysate-pulsed antigen-presenting cells. Non-TG, non-transgenic; TG, transgenic.

Discussion

We evaluated *in vitro* responses of mesenteric lymph node cells to physiologically relevant components of luminal contents, since mesenteric lymph nodes drain the diseased cecum and proximal colon in specific pathogen-free HLA-B27 transgenic rats. For these studies, we prepared lysates of cecal contents as described by Cong et al,[10] rather than attempting to culture intestinal bacteria for the following reasons: 1) the lysates contain bacterial products that are present at the site of maximal inflammation, 2) not all enteric bacteria can be cultured, and 3) culturing can change bacterial antigen expression.

We showed that cecal bacterial lysate can stimulate significantly higher amounts of those cytokines generally associated with T_H1 responses including IFN-γ, IL-12 and TNF from unseparated mesenteric lymph node cells collected from specific pathogen-free transgenic rats than from non-transgenic littermates. These results correlated with cecal mRNA expression of the same cytokines in colitic specific pathogen-free transgenic rats, which is in agreement with and extends a previous report by Rath et al.[3] We therefore conclude that *in vitro* production of proinflammatory cytokines by draining mesenteric lymph node cells stimulated with commensal cecal bacterial lysates correlates with mucosal cytokine responses in the diseased cecum. These results, and our failure to detect IFN-γ, IL-12 or TNF in cultures of mesenteric lymph node cells from germ-free transgenic rats, indicate that cecal bacteria and their products can induce these proinflammatory cytokines in mesenteric lymph nodes and cecal tissues of disease-susceptible specific pathogen-free HLA-B27 transgenic rats.

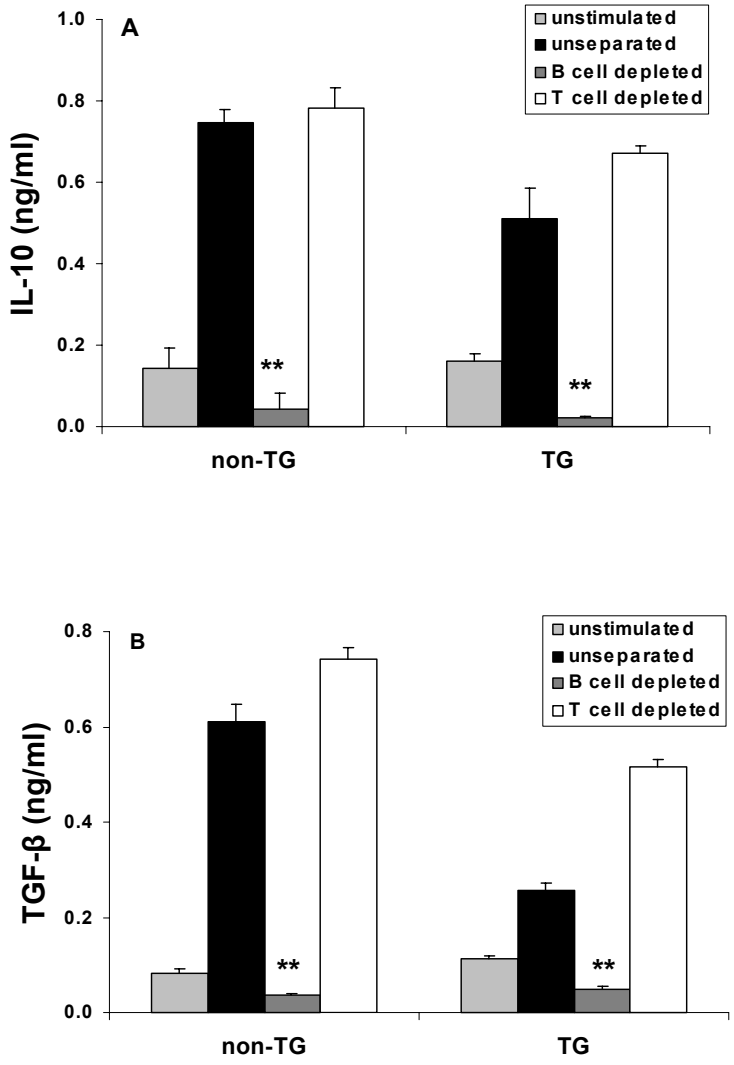

Figure 7. Cytokine levels in mesenteric lymph node cell cultures from four to five month old specific pathogen-free non-transgenic and transgenic rats. (A) IL-10 and (B) TGF-β production by mesenteric lymph node cells which were either unseparated, T cell depleted, or B cell depleted, then stimulated with 10 μg/ml of cecal bacterial lysate from non-transgenic rats. Cytokine levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants and are representative of seven (IL-10) or three (TGF-β) separate experiments. ** $p < 0.01$ versus unseparated mesenteric lymph node cell supernatants. Non-TG, non-transgenic; TG, transgenic.

T cell-depletion in our studies resulted in complete loss of IFN-γ responses by mesenteric lymph node cells from transgenic rats. Furthermore, CD4 T cell depletion of transgenic mesenteric lymph node cells abrogated IFN-γ production, whereas $CD8^+$ cell depletion had no effect. In addition, $CD4^+$-enriched mesenteric lymph node cells from transgenic but not from non-transgenic rats produced IFN-γ after stimulation with cecal bacterial lysate pulsed antigen-presenting cells. Combined, these results indicate that cecal bacteria and their products stimulate mesenteric lymph node-derived CD4 T cells to produce IFN-γ in specific pathogen-free transgenic rats. The disease-inducing capacity of these CD4 T cells was demonstrated by the ability of lymph node-derived $CD4^+$ cells from colitic specific pathogen-free transgenic donor rats to transfer colitis into specific pathogen-free nude transgenic recipients, which do not develop disease in the absence of T cells.[17] MHC class I molecules such as HLA-B27 are generally thought to activate

$CD8^+$, not $CD4^+$ T cells. However, $CD8^+$ T cells were not essential to the pathogenesis of colitis in HLA-B27 transgenic rats.[18]

While the role of the HLA-B27 molecule in development of inflammatory diseases has not been identified, a variety of plausible explanations have been proposed, based on the ability of HLA-B27 to activate $CD4^+$ T cells. Recent reports described $CD4^+$ T cells that recognized unusual forms of HLA-B27, such as heterodimers that lack an associated peptide, altered three dimensional structure of HLA-B27, or HLA-B27 homodimers.[19] HLA-B27 homodimers that are capable of binding peptide and thus taking on MHC class II-like conformation have been identified.[20] Moreover, MHC class I-restricted $CD4^+$ T cells derived from MHC class II–deficient mice induced colitis in congenic immunodeficient mice.[21] Any or all of the proposed mechanisms could explain the link between HLA-B27 expression and development of inflammatory disease in the transgenic rat model that we have employed.

Interestingly, in our studies, IFN-γ production by transgenic mesenteric lymph node cells was significantly reduced after B cell depletion, indicating either that B cells provide help for IFN-γ production by mesenteric lymph node-derived CD4 T cells through antigen-presenting cell activity or, less likely, that these cells produce IFN-γ. In a comprehensive analysis reported by Harris et al, B cells, like T cells, could be divided into subsets (designated Be1 and Be2).[22] Be1 cells, but not Be2 cells produce IFN-γ. In addition, different B cell subsets have the capacity to influence cytokine production by $CD4^+$ T cell subsets.[22] Furthermore, lower levels of IFN-γ were detected in spleen cell cultures of LCMV-infected B cell deficient mice compared to B cell replete mice after *in vitro* antigen stimulation.[23]

IL-12 production in response to the optimal concentration of cecal bacterial lysate was reduced by 80% in T cell-depleted mesenteric lymph node cell cultures, indicating that IL-12 production is T cell-dependent. Although IL-12 is mainly produced by antigen-presenting cells such as macrophages and dendritic cells, activated T cells can stimulate IL-12 production by antigen-presenting cells.[24]

IFN-γ, IL-12 and TNF were produced by cecal bacterial lysate-stimulated transgenic mesenteric lymph node cells, but not by non-transgenic mesenteric lymph node cells. In addition, IFN-γ, IL-12 and TNF were not detected in supernatants of lysate-stimulated mesenteric lymph node cell cultures from germ-free rats. Thus, the production of these three cytokines is dependent both on the genetic susceptibility of the host and on prior *in vivo* stimulation by commensal intestinal bacteria or the presence of colonic inflammation.

Of potential relevance to immunoregulation, we observed that cecal bacterial lysate-stimulated unseparated mesenteric lymph node cells from non-transgenic rats produced significantly more IL-10 and TGF-β relative to their transgenic littermates, whereas the production of another immunoregulatory molecule, PGE_2, was not significantly different in supernatants from lysate-stimulated transgenic and non-transgenic mesenteric lymph node cells. IL-10 is an immunoregulatory cytokine, produced by T_R1 and also by B cells and dendritic cells, with the ability to prevent the development of colitis. IL-10 inhibits antigen-specific proliferation and cytokine production by T_H1 lymphocytes and has downregulatory effects on antigen-presenting cells such as suppression of macrophage activation and IL-12 production.[25-27] The important anti-inflammatory effect of IL-10 is demonstrated by the observation that severe inflammatory disease develops in $IL\text{-}10^{-/-}$ mice. The role of commensal bacteria in this model is underscored by the observation that germ-free $IL\text{-}10^{-/-}$ mice do not develop colitis.[28-29] IL-12 and IFN-γ production in response to *in vitro* LPS stimulation is elevated in spleen cells from $IL\text{-}10^{-/-}$ compared to wild-type control mice.[30] Several *in vivo* studies have shown that parenteral administration of IL-10 or IL-10-producing T cells could prevent colitis in models of chronic

intestinal inflammation.[31-35] However, parenterally administered recombinant IL-10 appeared to have limited capacity to reverse established experimental colitis[11] or human Crohn's disease.[36] A local mucosal delivery of IL-10 by genetically engineered *Lactococcus lactis* or by adenoviral vectors encoding IL-10 was able to reverse colitis in IL-$10^{-/-}$ mice and DSS-induced colitis.[37-38]

TGF-β is another immunoregulatory molecule, which is produced by antigen-presenting cells and by T_H3/T_R1 lymphocytes, whereas TGF-β receptors are expressed on a variety of cells.[39] Of significant interest, we show here that cecal bacterial lysate-stimulated B cells, but not T cells, produce IL-10 and TGF-β. The importance of TGF-β in immune homeostasis is demonstrated by the fact that TGF-β deficient mice die within five weeks of severe multiple organ inflammation.[40] $CD4^+$ $CD25^+$ T cells exert immunosuppression by a cell-cell interaction involving cell surface TGF-β.[41] TGF-β is important for the regulation of intestinal inflammation. Intranasal administration of a TGF-β-containing plasmid prevented TNBS-induced colitis.[42]

Our results show that the levels of IL-10 and TGF-β in supernatants of unseparated mesenteric lymph node cell cultures from either germ-free or specific pathogen-free rats correlated with the percentage of B cells, and that neither IL-10 nor TGF-β were detected in B cell-depleted mesenteric lymph node cell cultures. B cells display several immune functions, such as production of immunoglobulins and cytokines, presentation of antigens, and potentiation of T cell responses.[19,43] B cells also have immunoregulatory functions,[44-46] and murine B-1 B cells have been shown to produce IL-10 after LPS stimulation.[47] Mizoguchi et al[48] demonstrated that IL-10-producing B cells are protective, since B cell/TCRα double deficient mice had more colitis than TCRα deficient mice with competent B cells. Also, transfer of B cells from IL-10/TCRα double deficient mice was unable to suppress chronic intestinal inflammation in B cell/TCRα double deficient mice.[48] Similarly, specific pathogen-free Gαi$2^{-/-}$ mice that develop colitis lack IL-10 producing B cells.[49] IL-10-producing B cells can also prevent arthritis.[50]

In our study mesenteric lymph node-derived B cells also produce TGF-β in both transgenic and non-transgenic rats, housed in either specific pathogen-free or germ-free conditions. Earlier studies have shown that normal B cells can produce TGF-β, which limits their own clonal expansion and differentiation.[51] An immunoregulatory function of TGF-β-producing B cells was shown by Tian et al in a murine model of autoimmune diabetes in which activated B cells produced TGF-β.[52] These B cells induced apoptosis of lymphocytes and prevented the development of autoimmune responses after co-transfer of B cells with disease-inducing T cells.

The bacterial components that stimulate cytokine production in our studies are not defined but are likely to be extremely heterogeneous, including LPS and PG-PS complexes that stimulate polyclonal responses by T cells and B cells as well as bacterial antigens that induce antigen-specific responses. While we have not identified the nature of the components that induce the responses evaluated here, we predict that bacterial products activate B cells in a polyclonal fashion via TLR as has been demonstrated.[53] In support of this view, we have found that LPS induces IL-10 and TGF-β, but not IFN-γ, in mesenteric lymph node cell cultures from transgenic and non-transgenic rats (data not shown). In addition, we propose that bacterial antigens, presented by antigen-presenting cells *in vitro*, restimulate T cells that have previously responded, *in vivo*, to the same bacterial antigens. A future direction of these studies is to further identify the effects of the different components of bacterial lysates.

In summary, our study indicates that mesenteric lymph node cells produce an array of cytokines in response to *in vitro* stimulation with normal, physiologically relevant cecal bacteria and their products. $CD4^+$ mesenteric lymph node cells from colitis-susceptible HLA-B27 transgenic rats, maintained in specific pathogen-free conditions, produce cytokines that are found in association with T_H1 immune responses. B cells, from both HLA-B27 transgenic rats and their

non-transgenic littermates, maintained in specific pathogen-free or germ-free conditions, produce IL-10 and TGF-β, cytokines that are often associated with protective immune response. Thus, our results reveal the diversity of responses of T cells and of B cells that can be activated by components of commensal bacteria in a rodent model of chronic intestinal inflammation.

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CD4$^+$ T lymphocytes mediate colitis induced by non-pathogenic *Bacteroides vulgatus* in HLA-B27 transgenic rats

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Frank Hoentjen[1,2], Susan L. Tonkonogy[3]
Levinus A Dieleman[1], Bi Feng Qian[1], Bo Liu[1]
Joel D Taurog[4], and R. Balfour Sartor[1]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

[3]College of Veterinary Medicine
North Carolina State University, Raleigh, USA

[4]Southwestern Medical Center
University of Texas, Dallas, USA

Submitted

Abstract

Introduction and aims

HLA-B27/β2 microglobulin transgenic rats develop spontaneous colitis when raised under specific pathogen-free conditions, whereas transgenic rats maintained under germ-free housing conditions fail to develop intestinal inflammation. Specific pathogen-free HLA-B27 transgenic *rnu/rnu* rats, which are congenitally athymic, remain disease-free in the absence of T lymphocytes. Taken together these results indicate that commensal intestinal bacteria and T cells are pivotal for the development of colitis in transgenic rats. Gnotobiotic transgenic rats monoassociated with *B. vulgatus* develop colitis. However, it is not known if T cells are also required in the induction of colitis by a single bacterial strain, such as *B. vulgatus*. The aim of this study was therefore to investigate the role of T cells in the development of colitis in *B. vulgatus*-monoassociated HLA-B27 transgenic rats.

Methods

HLA-B27 transgenic *rnu/rnu* and *rnu/+* rats where monoassociated with *B. vulgatus* for 8-12 weeks. $CD4^+$ T cells from mesenteric lymph nodes of *B. vulgatus* monoassociated *rnu/+* transgenic donor rats were transferred into *B. vulgatus* monoassociated *rnu/rnu* transgenic recipients. Mesenteric lymph node cells were collected and stimulated *in vitro* with *B. vulgatus*. Cecal and colonic tissue was collected for histology, MPO and cytokine analysis.

Results

B. vulgatus monoassociated *rnu/+* rats showed higher histologic inflammatory scores and elevated cecal and colonic IFN-γ mRNA, MPO, and cecal IL-1β levels, compared to *rnu/rnu* transgenic rats which did not contain T cells. After transfer of $CD4^+$ cells from colitic *B. vulgatus* monoassociated *rnu/+* transgenic donor rats into *B. vulgatus* monoassociated *rnu/rnu* transgenic recipients, colitis developed that was accompanied by *B. vulgatus*-induced IFN-γ responses by mesenteric lymph node cells and inflammatory parameters similar to *rnu/+* transgenic rats.

Conclusions

These results show that T cells are required, and $CD4^+$ cells are sufficient, for the development of colitis in HLA-B27 transgenic rats monoassociated with the non-pathogenic bacterial strain *B. vulgatus*.

Introduction

The important role of commensal intestinal bacteria in the pathogenesis of experimental colitis and human IBD has been emphasized by numerous recent studies. This has been demonstrated in several animal models of experimental colitis, in which the susceptible host develops spontaneous colitis in the presence of non-pathogenic intestinal organisms.[1] In a well characterized animal model of inflammation, HLA-B27/β2 microglobulin transgenic rats expressing high copy numbers of the human HLA-B27 gene develop spontaneous colitis, gastritis and arthritis by three months of age when raised under specific pathogen-free conditions.[2] Commensal bacteria of the intestine tract have been extensively implicated in development of disease. Treatment of transgenic rats with broad spectrum antibiotics is effective for preventing the onset and also for ameliorating established disease.[3] In addition, when HLA-B27 rats are raised in a germ-free environment they fail to develop intestinal inflammation and arthritis whereas colitis and gastritis develop within one month after transfer to a specific pathogen-free environment.[4,5] Although the results of numerous studies have been reported, the mechanisms underlying intestinal inflammation in HLA-B27 rats remain unclear. Disease does not develop in transgenic *rnu/rnu* rats, which are congenitally athymic and therefore lack conventional T lymphocytes, thus providing direct evidence that T cells are required for development of colitis in this model. Even if *rnu/rnu* transgenic rats carry the disease-prone B27 transgenic locus they are protected from disease, whereas the presence of T cells in their heterozygous *rnu/+* transgenic littermates coincides with development of colitis under specific pathogen-free conditions.[6] The critical role for T cells in inducing intestinal inflammation in this model was further emphasized by the development of colitis by after T cells had been transplanted into specific pathogen-free *rnu/rnu* HLA-B27 transgenic recipients, with $CD4^+$ T cells being more efficient than $CD8^+$ T cells in transferring disease.[6]

Most studies in HLA-B27 transgenic rats are carried out under specific pathogen-free conditions. However, monoassociation of HLA-B27 rats would provide a model to efficiently explore the role of T cells in the induction of colitis by a single bacterial strain. Studies from our laboratory have previously shown that monoassociation of HLA-B27 transgenic rats with *B. vulgatus*, a Gram-negative non-pathogenic microorganism, leads to induction and perpetuation of colitis in the genetically susceptible host. In HLA-B27 transgenic rats, *B. vulgatus* preferentially induces colitis within one month after monoassociation,[7] however, no inflammation results after monoassociation with an *E. coli* isolated from a Crohn's disease patient.[7] The ability of *B. vulgatus* to induce colitis was first described using carrageenan-fed guinea pigs as the model system.[8] Furthermore, *Bacteroides* species were identified among the bacteria that preferentially expanded in HLA-B27 transgenic rats after the creation of a blind cecal loop, which resulted in development of severe cecal inflammation.[9] To study the role of T cells in the chronic inflammation induced by *B. vulgatus*, we compared gnotobiotic HLA-B27 transgenic *rnu/rnu* and *rnu/+* rats after monoassociation with this single bacterial strain.

In the present study, we showed that *B. vulgatus* induced colitis in previously germ-free transgenic *rnu/+* but not in *rnu/rnu* transgenic rats. Furthermore, disease developed in *rnu/rnu* transgenic recipients after transfer of activated $CD4^+$ T cells from *B vulgatus* monoassociated *rnu/+* transgenic rats. These data provide clear evidence that T cells are required for the induction of colitis by *B. vulgatus* in *rnu/rnu* transgenic rats. Moreover, the results show that $CD4^+$ cells alone can generate the responses we observed; $CD8^+$ cells are not required.

Materials and methods

Animals

We used germ-free HLA-B27 transgenic rats (of the 33-3 line)[2,10] and their non-transgenic littermates that were either homozygous or heterozygous for the *nu* allele (*rnu/rnu*, or *rnu/+* respectively).[11,12] *Rnu/rnu* rats are athymic, whereas heterozygous *rnu/+* rats are euthymic and have normal T cell function.[6] All germ-free animals were monoassociated with *B. vulgatus* in the Gnotobiotic Animal Core of the Center for Gastrointestinal Biology and Disease at the College of Veterinary Medicine, NC State University in Raleigh, NC. Rats used for these experiments were four to eight months old, and animals were monoassociated with *B. vulgatus* for 8-12 weeks. Presence or absence of the HLA-B27 transgene was determined by PCR using DNA isolated from tail clippings, as described previously. All studies were approved by University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Experimental design

Germ-free *rnu/rnu* and *rnu/+* HLA-B27 transgenic rats were monoassociated for 8–12 weeks with *B. vulgatus* by oral and rectal swabbing with a slurry of fecal material from *B. vulgatus* monoassociated rats. In separate experiments, we transferred 10^7 mesenteric lymph node $CD4^+$ T cells derived from *rnu/+* HLA-B27 transgenic rats monoassociated with *B. vulgatus* intraperitoneally into *B. vulgatus* monoassociated *rnu/rnu* transgenic recipients, as described below. After seven to nine weeks, the rats were killed and mesenteric lymph node cells were collected for flow cytometry and cell cultures. Cecal and colonic tissue was collected for MPO, cecal cytokines, and histology, as described below. *B. vulgatus* derived from a guinea pig with carrageenan-induced colitis was a kind gift from Dr. A. B. Onderdonk, Harvard University, Cambridge, MA. *B. vulgatus* monoassociation was confirmed by anaerobic and aerobic cultures, as well as by Gram staining.

CD4+ T cell transfer

Donor cells were isolated from mesenteric lymph nodes from *rnu/+* HLA-B27 transgenic rats monoassociated with *B. vulgatus* for at least four weeks. Mesenteric lymph node cells were isolated by gentle teasing, washed twice, and then $CD4^+$ T cells were obtained by negative selection using anti-rat CD45RA and anti-rat CD8a antibody coated microbeads and columns designed for cell depletion according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA), as described previously.[13] Subsequently, 10^7 $CD4^+$ T cells were transferred intraperitoneally into *rnu/rnu* transgenic recipients monoassociated with *B. vulgatus*. At the time of transfer, recipients had been monoassociated for at least two weeks.

Histology

Colons and ceca were fixed and stained as previously described.[5,14] A validated histologic inflammatory score ranging from 0 to 4 was used for blinded evaluation.[5,14]

Preparation of bacterial lysates

Bacterial lysates were prepared as described previously.[13,15] Briefly, cecal contents, pooled from several *B. vulgatus* monoassociated non-transgenic rats, were disrupted using glass beads to lyse the bacteria. The lysate was then filtered through a 0.22 μm syringe filter.[13] A single colony of murine *E. coli*, isolated from specific pathogen-free normal C57BL/6 mice was cultured in Brain-Heart Infusion broth for 48 hours and the resulting bacteria were used to prepare a lysate

preparation as described above. Sterility of lysates was confirmed by aerobic and anaerobic bacterial culture.

Mesenteric lymph node cell cultures

Mesenteric lymph nodes were removed from the *rnu/rnu* and *rnu/+* transgenic rats, and single cell suspensions were prepared by gentle teasing. Unseparated mesenteric lymph node cells were washed twice and $4x10^5$ cells were cultured in 96 well flat bottom microplates (Costar 3595), in 0.2 ml complete medium (RPMI 1640 plus 5% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, $5x10^{-5}$ M 2-mecaptoethanol, and 50 μg/ml gentamicin) for three days. Cells were stimulated with different concentrations of *B. vulgatus* or *E. coli* lysate, as indicated. Culture supernatants were collected and stored at –20°C.

Flow cytometry

Mesenteric lymph node cells were evaluated by flow cytometry using the following fluorochrome labeled or unlabeled reagents: ME-1 (anti-HLA-B27) hybridoma culture supernatant (ATCC Rockville, MD); FITC labeled goat anti-mouse IgG (γ-chain specific) antibody (Southern Biotechnology, Birmingham, AL) to detect binding of the unlabeled ME-1; FITC labeled goat anti-rat IgG (H + L) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) to identify B cells; PE-anti-rat CD4 (clone W3/25), and FITC anti-rat CD8 (clone OX-8), (Caltag, Burlingame, CA); and FITC anti-rat TCRαβ (clone R73) (BD Biosciences Pharmingen) to identify T cell subpopulations.

Cytokine measurements

We used the following commercially available ELISA reagents, as previously described, to measure IFN-γ and IL-10 in culture supernatants.[13] Unlabeled polyclonal anti-IFN-γ antibody and biotin-labeled monoclonal anti-IFN-γ antibody (clone DB-1) (Biosource International, Camarillo, CA), unlabeled monoclonal anti-rat IL-10 antibody (clone A5-7) and biotin-labeled monoclonal anti-rat IL-10 antibody (clone A5-6) (BD Biosciences Pharmingen, San Diego, CA), and horseradish peroxidase labeled Streptavidin (BD Biosciences Pharmingen). Standard curves were generated using recombinant cytokines (BD Biosciences Pharmingen).

Cytokine mRNA expression in cecal tissues

RNA was isolated from snap frozen cecal tissue using TRIzol (Invitrogen, Carlsbad, CA), reverse transcribed (1 μg RNA), and amplified using primers specific for rat IFN-γ as described previously.[13,16] The PCR products (7 μl) were subjected to electrophoresis on 2% agarose gels containing gel Star fluorescent dye (FMC, Philadelphia, PA). Fluorescence staining was captured using an Alpha Imager 2000 (AlphaInnotech, San Leandro, CA). The IFN-γ mRNA was quantified by densitometry, and the ratio to β-actin mRNA was calculated.

Cecal IL-1β analysis

For measurement of cecal IL-1β, tissue from the distal cecum was homogenized in the presence of protease inhibitors.[17] IL-1β was determined in the supernatant according to the manufacturer's instructions (National Institute for Biological Standards and Controls, South Mimms, UK).

MPO assay

Ceca were homogenized as described in the previous section. Subsequently, MPO activity (units per gram of cecal tissue) was quantified, as described previously.[14,18]

Statistical analysis

Concentrations of the various cytokines are expressed as mean ± standard error as measured in triplicate culture supernatants. Data were analyzed using the paired Student's t-test, and a P value of < 0.05 was considered statistically significant.

Results

Colitis in *B. vulgatus* monoassociated transgenic rats

Transgenic *rnu/+* rats monoassociated with *B. vulgatus* for 8-12 weeks demonstrated a mild histologic colitis in both cecum and colon (figures 1A, 2). This inflammation is characterized by mononuclear cell infiltration of the intestinal mucosa with crypt hyperplasia. In contrast, transgenic *rnu/rnu* rats housed under identical conditions did not show microscopic signs of colitis (figures 1B, 2). When $CD4^+$ T cells derived from colitic *B. vulgatus* monoassociated *rnu/+* transgenic rats were transferred into *B. vulgatus* monoassociated *rnu/rnu* transgenic rats, the recipients developed mild colitis in the cecum and colon. This inflammation was very similar in appearance and severity compared to that observed in the intestinal tract of transgenic *rnu/+* rats monoassociated with *B. vulgatus* (figures 1C and 2).

Identification of mesenteric lymph node cell populations

Mesenteric lymph nodes from *B. vulgatus* monoassociated transgenic *rnu/+* rats with colitis were larger and contained over 5-fold more cells than those from transgenic *rnu/rnu* rats without disease (table 1). Mesenteric lymph nodes from transgenic *rnu/+* rats contained 5-fold more $CD4^+$ T cells than $CD8^+$ T cells (table 1). Most of the transgenic *rnu/rnu* mesenteric lymph node cells expressed immunoglobulin, a surface marker of B cells, whereas transgenic *rnu/+* mesenteric lymph node cells contained 2-3 fold fewer B cells compared to those from *rnu/rnu* transgenic rats.

Donor mesenteric lymph node cells, enriched for $CD4^+$ T cells, were 98.1 ± 0.2% $CD4^+$ cells (data not shown). After adoptive transfer of $CD4^+$ T cells, the total number of unseparated mesenteric lymph node cells as well as the proportion of $CD4^+$ cells was significantly higher, while the proportion of surface Ig expressing cells was lower in *rnu/rnu* transgenic recipients compared to *rnu/rnu* transgenic rats that did not receive $CD4^+$ T cells. All animals identified as HLA-B27 transgenic by PCR analysis of tail DNA also expressed HLA-B27 on mesenteric lymph node cells.

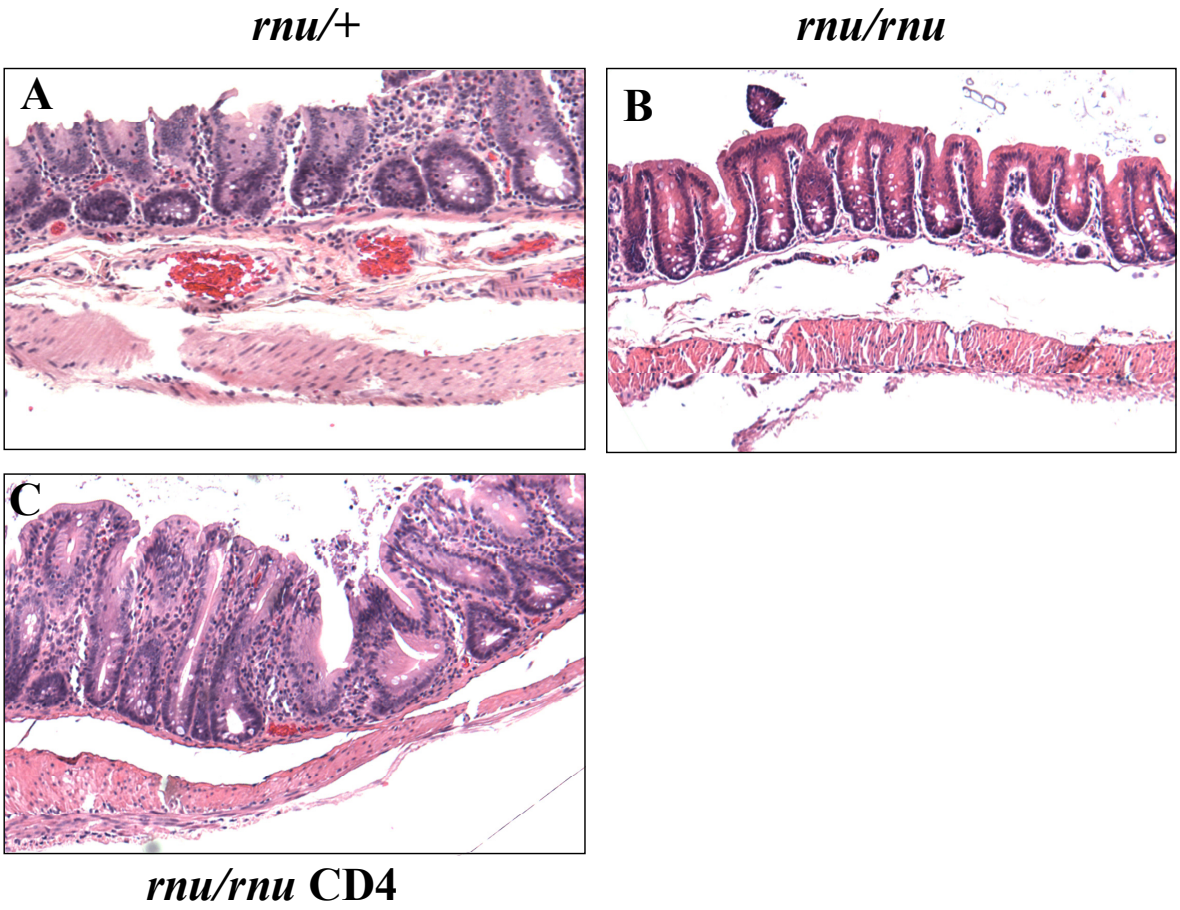


Figure 1. Representative photomicrographs of tissue sections (x10) from cecal tissue of six to eight month old (A) *B. vulgatus* monoassociated transgenic *rnu/+* rats; (B) *B. vulgatus* monoassociated transgenic *rnu/rnu* rats; (C) *B. vulgatus* monoassociated transgenic *rnu/rnu* rats after $CD4^+$ T cell transfer.

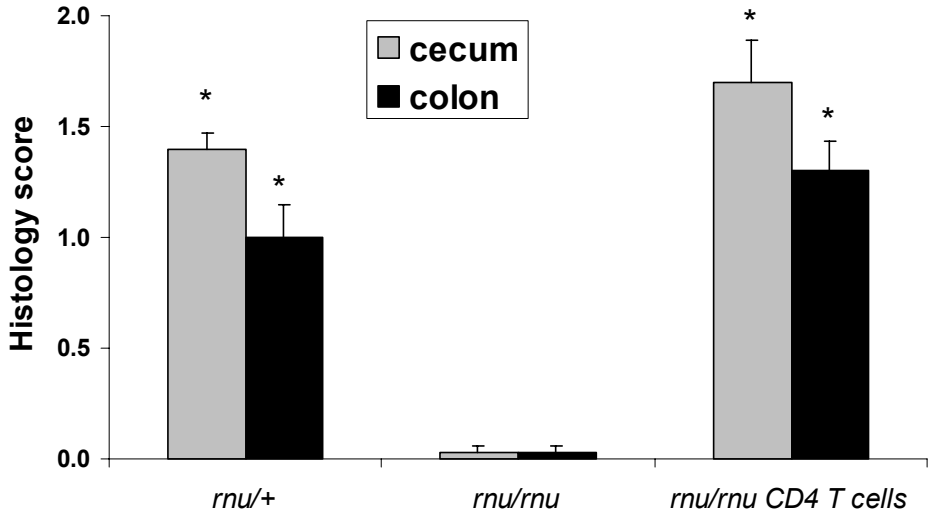


Figure 2. Blinded cecal and colonic inflammatory scores in *B. vulgatus* monoassociated transgenic *rnu/+* and transgenic *rnu/rnu* rats, and *B. vulgatus* monoassociated transgenic *rnu/rnu* rats after $CD4^+$ T cell transfer. Monoassociated rats were killed 8-12 weeks after *B. vulgatus* monoassociation. Values represent mean ± SEM of cecal or colonic histology scores (0-4). Each group consisted of six or seven rats. *$P < 0.001$ versus transgenic *rnu/rnu* rats.

	***B. vulgatus* monoassociated HLA- B27 transgenic rats**		
	rnu/rnu	*rnu/+*	*rnu/rnu* CD4 T cells
	n=7	n=7	n=6
Total Cells (x 10^6)	19 ± 2	100 ± 8[a]	48 ± 6[b]
Percent			
TCRαβ	1.9 ± 0.6	63.0 ± 3.0[a]	9.7 ± 1.4[b]
CD4	2.8 ± 0.3	51.1 ± 1.9[a]	9.6 ± 1.4[b]
CD8	1.5 ± 0.1	10.2 ± 0.7[a]	1.3 ± 0.2
Surface Ig	93.5 ± 0.7	36.4 ± 3.2[a]	81.7 ± 1.2[a]
HLA-B27	97.3 ± 0.7	98.1 ± 0.1	96.5 ± 0.7

Table 1. Cell numbers and cell subpopulations in mesenteric lymph nodes of *B. vulgatus* monoassociated *rnu/rnu*, *rnu/+*, and *rnu/rnu* recipients of CD4 T cells from *rnu/+* HLA-B27 *B. vulgatus* monoassociated transgenic rats. Values represent mean ± SEM of total cells or of percentages of lymphoid cell subpopulations. [a] $p<0.001$, [b] $p<0.01$ versus *rnu/rnu* transgenic rats.

Mucosal cytokine and MPO production

IL-1β levels in cecal homogenates reflected the severity of cecal inflammation in *rnu/+* transgenic rats. Compared to *B vulgatus* monoassociated *rnu/rnu* transgenic rats, cecal tissue from *rnu/+* rats and from *rnu/rnu* $CD4^+$ T cell recipients contained significantly more IL-1β (figure 3A), with the latter group producing higher levels of cecal IL-1β than *rnu/+* rats ($P < 0.05$).

Cecal MPO showed the same pattern as cecal IL-1β with approximately 2-fold more MPO in cecal tissue from transgenic *rnu/+* rats and $CD4^+$ T *rnu/rnu* cell recipients compared to transgenic *rnu/rnu* rats, and higher MPO levels in the $CD4^+$ T cell *rnu/rnu* cell recipients compared to transgenic *rnu/+* rats (figure 3B).

Cecal and colonic IFN-γ mRNA expression

The IFN-γ/β actin mRNA ratio was significantly higher in colons from transgenic *rnu/+* rats and from *rnu/rnu* recipients of CD4 T cells compared to transgenic *rnu/rnu* rats ($P < 0.05$, figure 4A and 4B). The cecal IFN-γ/β actin mRNA ratio in *rnu/rnu* recipients of CD4 T cells was also significantly higher than ratio for cecal tissue from *rnu/rnu* animals.

Mesenteric lymph node cell cytokine production

Mesenteric lymph node cells isolated from either transgenic *rnu/rnu* or from transgenic *rnu/+* rats did not produce detectable levels of IFN-γ after stimulation with cecal lysates prepared from *B. vulgatus* monoassociated rats (figure 5A). Interestingly, when $CD4^+$ T cells were transferred into *rnu/rnu* transgenic recipients monoassociated with *B. vulgatus*, mesenteric lymph node cells stimulated with *B. vulgatus* lysate secreted significant amounts of IFN-γ (figure 5A). However, when mesenteric lymph node cells in all three groups were stimulated with *E. coli* lysates, no IFN-γ was detected (data not shown), suggesting that IFN-γ responses observed are specific for components of *B. vulgatus*. Mesenteric lymph node cells from all three groups of rats produced IL-10 after stimulation with *B. vulgatus* lysate, indicating that the mesenteric lymph node cell obtained from each of the rats are capable of responding *in vitro* to components of the bacterial lysate.

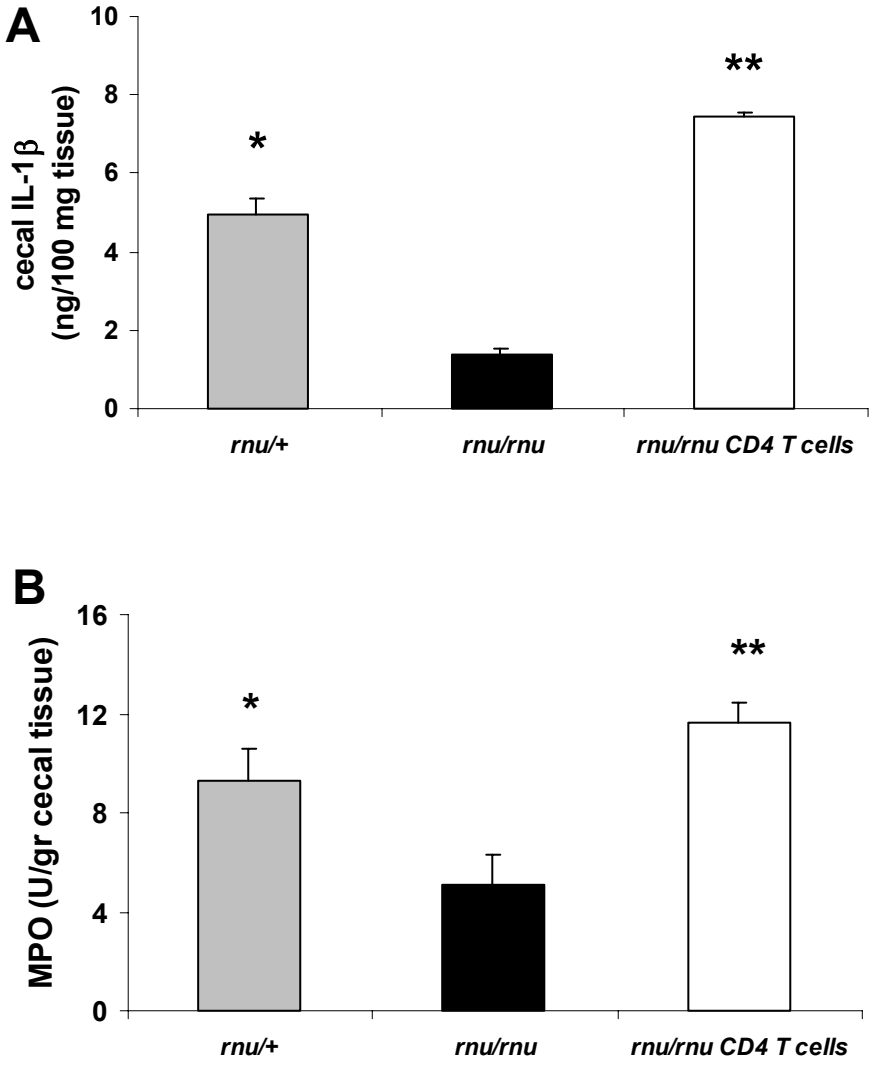
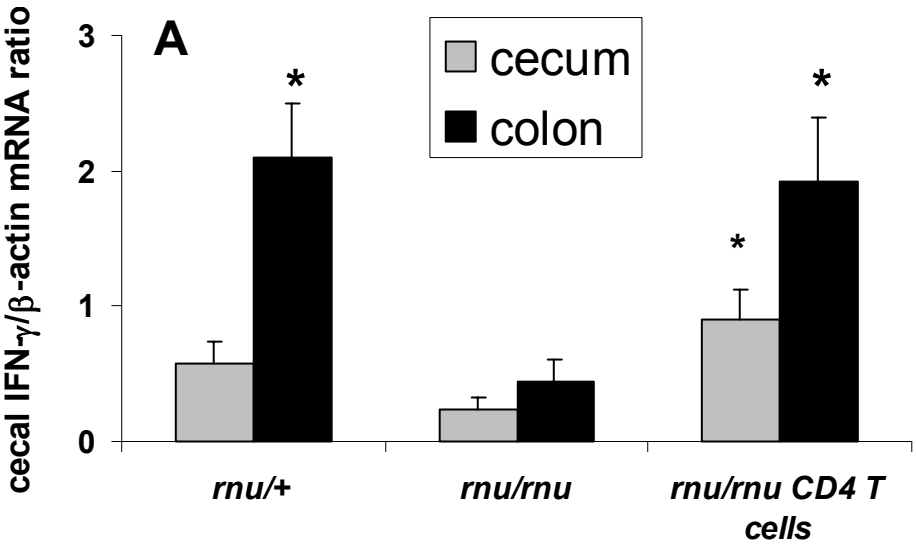


Figure 3. IL-1β (A) and MPO (B) in cecal homogenates from *B. vulgatus* monoassociated transgenic *rnu/+* and transgenic *rnu/rnu* rats, and *B. vulgatus* monoassociated transgenic *rnu/rnu* rats after $CD4^+$ T cell transfer. Data represent mean ± SEM of values obtained from 6-7 rats per group *P < 0.05 versus transgenic *rnu/rnu* rats. **P < 0.05 versus transgenic *rnu/+* rats.

Discussion

B. vulgatus has been incriminated in the induction and perpetuation of colitis. It is an anaerobic Gram-negative bacterial strain that resides in the colon in high concentrations. *B. vulgatus* is a non-pathogenic bacterium; it does not induce colitis in wild-type rats. However, HLA-B27 transgenic littermates that are genetically susceptible hosts develop intestinal inflammation after monoassociation with *B. vulgatus*.[7]



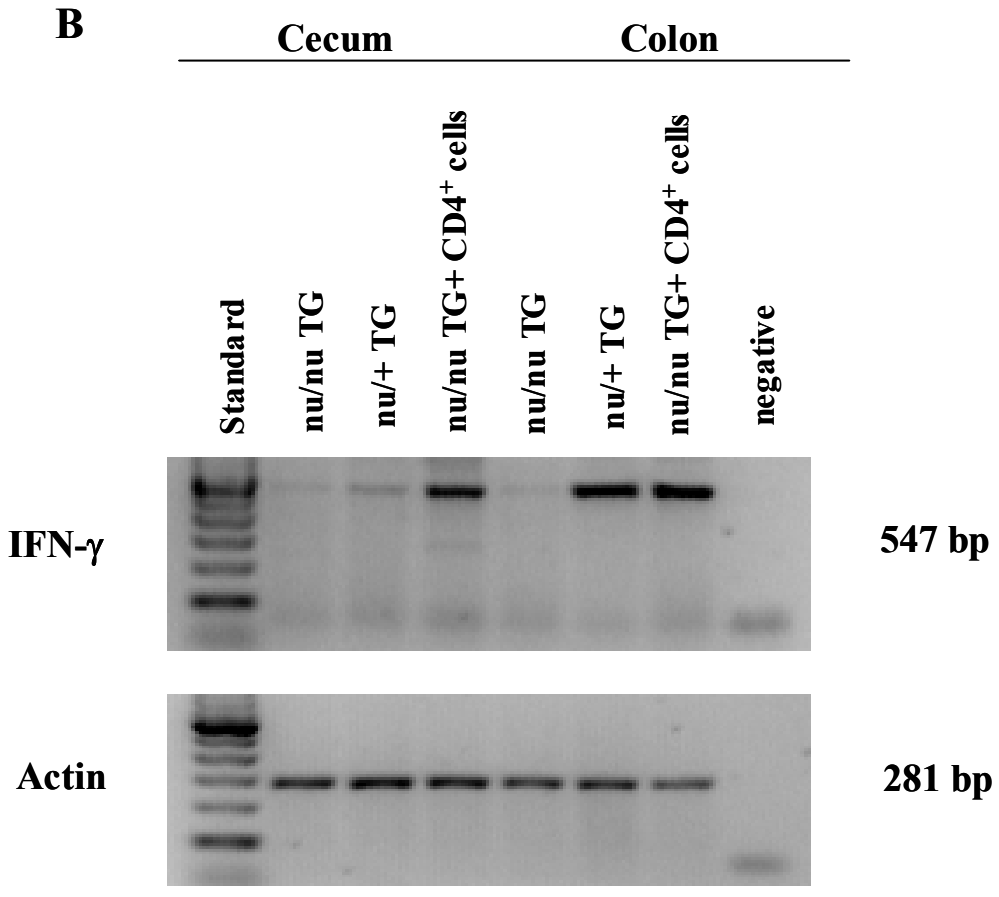


Figure 4. Expression of IFN-γ mRNA in cecal and colonic tissues from *B. vulgatus* monoassociated transgenic *rnu/+* and transgenic *rnu/rnu* rats, and *B. vulgatus* monoassociated transgenic *rnu/rnu* rats after $CD4^+$ T cell transfer. (A) Results are expressed as the ratio of IFN-γ mRNA versus β-actin. * $P < 0.05$ versus transgenic *rnu/rnu* rats. (B) IFN-γ PCR products from cecal and colonic samples of one representative rat from each group. Non-TG, non-transgenic; TG, transgenic.

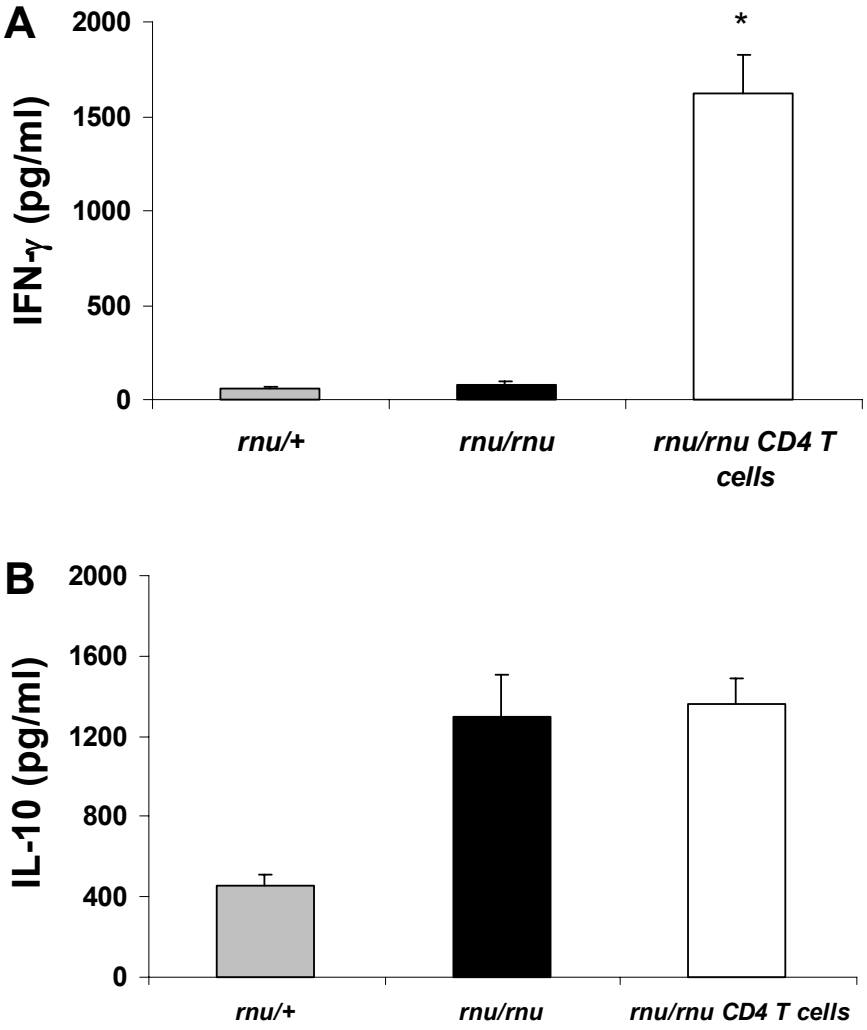


Figure 5. IFN-γ and IL-10 production in mesenteric lymph node cell cultures from *B. vulgatus* monoassociated transgenic *rnu/+* and transgenic *rnu/rnu* rats, and *B. vulgatus* monoassociated transgenic *rnu/rnu* rats after $CD4^+$ T cell transfer. Rats were killed 8-12 weeks after *B. vulgatus* monoassociation and mesenteric lymph node cells were stimulated with cecal bacterial lysate from *B. vulgatus* monoassociated non-transgenic *rnu/+* rats. IFN-γ (A) and IL-10 (B) were measured in triplicate culture supernatants by ELISA. Values represent mean values (pg/ml) ± SEM. *P < 0.005 versus transgenic *rnu/rnu* rats.

In the present study, we showed for the first time that T cells are required for the induction of intestinal inflammation in *rnu/rnu* transgenic rats monoassociated with non-pathogenic commensal *B. vulgatus*. This was shown by histology scores, cecal MPO and IL-1β, and colonic IFN-γ mRNA, that were higher in *rnu/+* transgenic rats compared to *rnu/rnu* transgenic rats. The level of histologic inflammation in *rnu/+* transgenic rats as detected in our study is similar to the histologic scores in transgenic rats that do not carry a nude allele, reported by Rath et al.[7] In that study, *B. vulgatus* monoassociated transgenic rats with normal T cell development showed average cecal histologic scores of 1.7 ± 0.2, compared to 1.4 ± 0.1 found in our study for *rnu/+* transgenic rats. Interestingly, we observed that *rnu/+* HLA B27 transgenic rats housed under specific pathogen-free conditions had consistently higher histologic inflammation scores compared to *rnu/+* transgenic rats that were monoassociated with *B. vulgatus* (data not shown). The complex intestinal microflora that colonizes the intestinal tract under specific pathogen-free conditions provides a strong antigenic stimulus for the genetically susceptible host resulting in a more severe intestinal inflammation than in *B. vulgatus*-monoassociated transgenic rats, with

likely synergistic effects between multiple bacterial strains. Monoassociation with *B. vulgatus* induces only mild inflammation, probably due to the lack of other disease-inducing microorganisms.

In the HLA-B27 transgenic rat model, Breban et al previously showed that *rnu/rnu* HLA-B27 transgenic rats housed under specific pathogen-free conditions do not develop colitis.[6] The results of our study extend those findings, confirming that T cells are also one of the critical components of the potentially less complex set of factors that are involved in development of colitis after colonization with a single microorganism. In several other animal models, T cells have been shown to be crucial to the development of inflammation. For example, SCID mice develop colitis upon transfer of naive T cells ($CD4^+$ T cells expressing high levels of CD45RB in the mouse).[19] The equivalent population of naive $CD4^+$ T cells in the rat, cells that express high levels of CD45RC, induced inflammation in the stomach and pancreas and also wasting disease in athymic rats.[20] T cells have also been shown to play a pivotal role in the colitis that develops in $IL\text{-}10^{-/-}$ mice,[21] $IL\text{-}2^{-/-}$ mice,[22] and CD3ε transgenic mice.[23] However, all studies mentioned have been performed under specific pathogen-free conditions. The requirement for T cells has not been demonstrated in a model of monoassociation with a non-pathogenic bacterial strain, as demonstrated in the present study.

It has been clearly shown that CD4, and not CD8 cells, are crucial for disease-induction in HLA-B27 transgenic rats. $CD4^+$ T cells were much more efficient than $CD8^+$ T cells in transferring disease into nude HLA-B27 rats.[6,24] In the present study, $CD4^+$ T cells were able to induce colitis in *B. vulgatus* monoassociated *rnu/rnu* transgenic rats that was similar in histologic severity to that of *B. vulgatus* monoassociated *rnu/*+ transgenic rats. We recently demonstrated that IFN-γ is produced by cecal bacterial lysate-stimulated mesenteric lymph node cells derived from specific pathogen-free HLA-B27 transgenic rats with severe colitis.[13] Depletion of $CD4^+$ cells, but not $CD8^+$ cells resulted in complete loss of IFN-γ secretion by mesenteric lymph node cells from transgenic rats. In addition, $CD4^+$-enriched mesenteric lymph node cells from transgenic but not from non-transgenic rats produced IFN-γ after stimulation with cecal bacterial lysate pulsed antigen-presenting cells.[13] Combined, these results indicate that cecal bacteria and their products stimulate mesenteric lymph node-derived $CD4^+$ T cells to produce IFN-γ in specific pathogen-free transgenic rats. In the present study, mesenteric lymph node cells from *B. vulgatus* monoassociated *rnu/*+ transgenic rats stimulated with *B. vulgatus* lysates did not produce detectable IFN-γ *in vitro*. In contrast, after transfer of $CD4^+$ T cells into *B. vulgatus* monoassociated *rnu/rnu* transgenic rats, mesenteric lymph node cells from recipients did secrete IFN-γ. The reason for this discrepancy is unclear. However, a possible explanation comes from studies of lymphoid cell homeostasis.[25] In a variety of model systems in which lymphocytes were transferred into lymphopenic hosts such as SCID mice, nude mice, nude rats, or $RAG^{-/-}$ mice, responses of the transferred cells were exacerbated compared to responses that would be seen in hosts with a normal complement of lymphocytes. One explanation for the apparently heightened responses of the transferred cells is that they proliferate until the lymphoid system reaches homeostasis, due in part to the lack of competition for cytokines and other factors that the cells require.[26] As shown in table 1, *rnu/rnu* rats are indeed lymphopenic. We postulate therefore that the lack of homeostatic control of lymphocytes numbers in the mesenteric lymph nodes of the *B. vulgatus* monoassociated *rnu/rnu* recipient rats allows proliferation and differentiation of the transferred *rnu/*+ $CD4^+$ cells. These *B. vulgatus* reactive mesenteric lymph node cells then produce IFN-γ after *in vitro* re-stimulation with *B. vulgatus* components.

While we did not detect IFN-γ after *B. vulgatus* lysate stimulation of mesenteric lymph node cells from monoassociated *rnu/*+ rats, colonic tissue isolated from these rats shows

expression of IFN-γ mRNA. It is possible that resident intraepithelial lymphocytes, a heterogeneous population of cells that have been shown to express IFN-γ mRNA and to produce this cytokine are the source of this activity.[27]

In addition, it should be noted that B cells and not T cells are likely to be the source of IL-10 secreted in culture supernatants of *B. vulgatus* lysate stimulated mesenteric lymph node cells (figure 5). In our previous report, we demonstrated that B cell depletion but not T cell depletion results in a failure of mesenteric lymph node cells from specific pathogen-free transgenic and non-transgenic rats to produce IL-10 after cecal bacterial lysate stimulation.[13] In the present study, *B. vulgatus* lysate stimulated mesenteric lymph node cells from *rnu/rnu* transgenic rats, which are comprised primarily of B cells, produce IL-10. The lower levels of IL-10 detected in culture supernatants of *B. vulgatus* lysate stimulated mesenteric lymph node cells from *rnu/+* transgenic rats correlate with the lower proportion of B cells in the mesenteric lymph nodes of these animals (table 1).

The molecular basis for the development of spondyloarthrophies in human patients that carry the HLA-B27 allele and for the occurrence inflammatory conditions in HLA-B27 transgenic rats has not yet been determined. However, recent reports implicate the unusual biochemical properties of this MHC Class I encoded molecule in disease development. The B27 heavy chain can take several different forms in addition to the classical heterodimer of the Class I encoded heavy chain non-covalently associated with β2 microglobulin that presents antigenic peptides to $CD8^+$ T cells.[28] One of the unusual forms results from homodimerization of two B27 molecules. An intriguing possibility that would explain the development of HLA-B27 associated disease was recently described by Kollnberger et al.[29] The results of their elegant study using tetramers of B27 homodimers indicate that these molecules are able to activate macrophages to produce TNF and nitric oxide, which would then establish a receptive environment for the development of inflammatory conditions.

In conclusion, results of the present study show that T cells are required for development of colitis in HLA-B27 transgenic rats monoassociated with the non-pathogenic *B. vulgatus*. Furthermore, disease could be transferred by activated $CD4^+$ T cells into *rnu/rnu* transgenic recipients, indicating that CD4-expressing cells are sufficient to generate the responses we measured. Studies in disease-susceptible gnotobiotic rodents monoassociated with disease-inducing commensal intestinal bacteria will help to unravel the complex microbial-host interactions that lead to chronic intestinal inflammation.

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Adoptive transfer of non-transgenic mesenteric lymph node cells induces colitis in athymic HLA-B27 transgenic nude rats

8

Frank Hoentjen[1,2], Susan L. Tonkonogy[3]
Bo Liu[1], R. Balfour Sartor[1], Joel D Taurog[4]
and Levinus A Dieleman[1]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

[3]College of Veterinary Medicine
North Carolina State University, Raleigh, USA

[4]Southwestern Medical Center
University of Texas, Dallas, USA

Submitted

Abstract

Introduction and aims

HLA-B27 transgenic rats develop spontaneous colitis when colonized with intestinal bacteria, whereas athymic *rnu/rnu* HLA-B27 transgenic rats remain disease free. The present study was designed to assess the relative contribution of both T cells and non-T cells in development of colitis after transfer of mesenteric lymph node cells into *rnu/rnu* HLA-B27 recipients lacking T cells.

Methods

Athymic non-transgenic and HLA-B27 transgenic recipients received either transgenic or non-transgenic mesenteric lymph node cells from *rnu/+* heterozygous donor rats that contain T cells. After eight weeks recipients were euthanized and mesenteric lymph node cells were collected for flow cytometry and *in vitro* stimulation with cecal bacterial lysate. Cecal and colonic tissues were collected for histologic scoring, and MPO and IL-1β were measured in cecal homogenates.

Results

HLA-B27 transgenic *rnu/rnu* recipients receiving either non-transgenic or transgenic mesenteric lymph node cells developed severe colitis and had higher cecal MPO and IL-1β levels, and their mesenteric lymph node cells produced more IFN-γ and less IL-10 after *in vitro* stimulation with cecal bacterial lysate compared to *rnu/rnu* non-transgenic recipients which remain disease free after receiving either transgenic or non-transgenic cells. Interestingly, proliferating donor transgenic T cells were detectable one week after adoptive transfer into *rnu/rnu* transgenic recipients but not after transfer into non-transgenic recipients.

Conclusions

T cells from either non-transgenic or transgenic donors induce colitis in *rnu/rnu* transgenic but not in non-transgenic rats, suggesting that activation of effector T cells by other cell types that express HLA-B27 is pivotal for the pathogenesis of colitis in this model.

Introduction

There is overwhelming evidence that IBD occur due to an overly aggressive immune response to commensal non-pathogenic bacteria in a genetically susceptible host. Many animal models of experimental colitis support this concept.[1] In these models, the genetically susceptible host develops colitis when housed under specific pathogen-free conditions, whereas the germ-free state prevents intestinal inflammation.

A well-characterized model of experimental colitis is the HLA-B27 transgenic rat model. HLA-B27 transgenic rats expressing the human MHC Class I gene, HLA-B27 and β2-microglobulin, develop spontaneous colitis in specific pathogen-free conditions.[2,3] Also a single strain of non-pathogenic commensal bacteria, *B. vulgatus*, is able to induce colitis in this model.[4,5] However, intestinal inflammation does not occur in germ-free animals[6] or after monoassociation with another commensal intestinal bacterial species, *E. coli.*[4] Moreover, mesenteric lymph node T cells produce IFN-γ in response to cecal bacterial lysates.[7] Taken together, these results suggest that commensal luminal bacteria play a crucial role in the induction and perpetuation of colitis in HLA-B27 transgenic rats.

T cells, and more specifically $CD4^+$ T cells, are required for the development of inflammation in HLA-B27 transgenic rats, as shown in cell transfer studies using athymic HLA-B27 transgenic rats. HLA-B27 transgenic rats homozygous for the *rnu* allele (*rnu/rnu*) are hairless and lack a developed thymus. *Rnu/rnu* HLA-B27 transgenic rats do not develop disease.[8] When transgenic $CD4^+$ T cells were transferred into *rnu/rnu* transgenic rats, intestinal inflammation developed.[8] We previously showed that activated $CD4^+$ T cells stimulated with cecal bacterial lysates are required for production of the T_H1 cytokines IFN-γ and IL-12.[7] These findings underline the importance of T cells, specifically $CD4^+$ cells, in the pathogenesis of colitis in the HLA-B27 transgenic model.

Several subsets of cells have been proposed to regulate immune responses to commensal luminal bacteria. Transfer of $CD4^+$ cells expressing high levels of one of the CD45 isoforms (designated $CD45RB^{hi}$ in the mouse; $CD45RC^{hi}$ in the rat) induced colitis in SCID mice[9], and gastritis, pancreatitis, and wasting disease in athymic rats.[10] In both models, the inflammatory processes could be attenuated by co-transfer of $CD4^+$ cells that express low levels of CD45RB or CD45RC. In addition, IL-10 producing B cells can suppress colitis mediated by $CD4^+TCR\alpha^-\beta^+$ T cells in TCRα deficient mice.[11] Thus, there are several distinct pathways for the regulation of the immune responses that mediate chronic intestinal inflammation.

In the HLA-B27 rat model, little is known about regulation of immune responses. We showed previously that B cells from both HLA-B27 transgenic rats and their non-transgenic littermates produce IL-10 and TGF-β, which are important regulatory cytokines.[7] Therefore, the present study was designed to assess whether co-transfer of mesenteric lymph node B cells and T cells from either non-transgenic or transgenic rats would induce or prevent intestinal inflammation in the *rnu/rnu* transgenic host. Furthermore, we investigated the role of the non-T cells that express HLA-B27 in induction of disease. Our results show that mesenteric lymph node cells from either non-transgenic or transgenic donors induce colitis in *rnu/rnu* HLA-B27 transgenic, but not in non-transgenic recipient rats. Furthermore, donor transgenic $CD4^+$ cells did not expand after adoptive transfer into *rnu/rnu* non-transgenic recipients. These findings suggest that activation of T cells by HLA-B27-expressing accessory cells, defined as non-T cells that either present antigen to T cells or perform co-stimulatory functions, is pivotal to the pathogenesis of colitis in this model.

Materials and methods

Animals

We used HLA-B27 transgenic F344 strain rats of the 33-3 line[2,12] and their non-transgenic littermates that were either homozygous or heterozygous for the *rnu* allele (*rnu/rnu*, or *rnu/+* respectively).[13,14] *Rnu/rnu* rats are athymic and hairless, whereas heterozygous *rnu/+* rats are euthymic and have normal T cell function.[8] Animals were all maintained under specific pathogen-free housing conditions at the University of North Carolina, Chapel Hill. Presence or absence of the HLA-B27 transgene was determined by PCR using DNA isolated from tail clippings. All studies were approved by University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Experimental procedure

Donor cells were obtained from mesenteric lymph nodes from *rnu/+* non-transgenic and transgenic rats. After obtaining single mesenteric lymph node cell suspensions by gentle teasing, the cells were washed twice and 10^7 unfractionated mesenteric lymph node cells were injected intravenously into the tail vein of four to eight month old *rnu/rnu* non-transgenic and transgenic recipients. After eight weeks, rats were euthanized. Cecal and colonic tissue and mesenteric lymph nodes were collected.

In separate experiments, donor mesenteric lymph node cells from *rnu/+* transgenic rats were labeled with CFSE (Molecular Probes, Eugene, OR) in order to monitor them after transfer, as described previously.[15,16] After obtaining single mesenteric lymph node cell suspensions by gentle teasing, the cells were washed twice and incubated with CFSE at 5 μM in 1xPBS for 15 minutes at room temperature. The labeling process was terminated by adding an equal volume of fetal calf serum. The cells were washed three times, and $1.5\text{-}2\text{x}10^7$ cells were injected intravenously into *rnu/rnu* transgenic or *rnu/rnu* non-transgenic recipients. Rats were euthanized on day two or day seven after injection, and flow cytometry was performed on recipient mesenteric lymph node and spleen cells, and recipient mesenteric lymph node cells were stimulated *in vitro* as described below.

Histology

Tissues from different sections of the intestinal tract (proximal, transverse, and distal colon, and cecum) were collected from each recipient. The tissues were fixed and stained as previously described.[3,17] Histologic scoring (range from 0-4) was based on well-validated criteria and carried out on blinded samples by one evaluator.[3,17]

Preparation of cecal bacterial lysates

Cecal bacterial lysates were prepared as described previously.[7,18] Briefly, cecal contents from several non-transgenic rats were disrupted using glass beads and the lysate was filter-sterilized (0.22 μm).[7] Sterility was confirmed by aerobic and anaerobic bacterial culture.

Mesenteric lymph node cell cultures

Mesenteric lymph nodes were removed from the *rnu/rnu* non-transgenic and transgenic recipients, and single cell suspensions were prepared, as described previously.[7] Unseparated mesenteric lymph node cells were washed twice and $4\text{x}10^5$ cells were stimulated with cecal bacterial lysate cultured in 96 well flat bottom microplates (Costar 3595), in 0.2 ml complete medium (RPMI 1640 plus 5% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium

pyruvate, $5x10^{-5}$ M 2-mercaptoethanol, and 50 μg/ml gentamicin) for three days, as described.[7] Cells were stimulated with concentrations of cecal bacterial lysate that we found to be optimal for the induction of different cytokines (50 μg/ml for stimulation of IFN-γ production, or 10 μg/ml for stimulation of IL-10 production). Culture supernatants were collected and stored at –20°C.

Flow cytometry

Mesenteric lymph node cell fractions were evaluated by flow cytometry using the following fluorochrome labeled or unlabeled reagents, as previously described.[7] For detection of HLA-B27-expressing cells, we used culture supernatants from the murine hybridoma, designated ME-1, obtained from ATCC (Rockville, MD), followed by either FITC labeled goat anti-mouse IgG (γ-chain specific) antibody (Southern Biotechnology, Birmingham, AL) or APC labeled rat anti-mouse IgG1 (BD Biosciences Pharmingen, San Diego, CA). For surface immunoglobulin positive B cells, we used FITC labeled goat anti-rat IgG (H + L) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). For CD4 and CD8, TCRαβ and CD45RC expressing cells we used PE-anti-CD4 monoclonal antibody (clone W3/25), and FITC anti-CD8 monoclonal antibody (clone OX-8), (Caltag, Burlingame, CA), FITC or PerCP anti-rat TCRαβ (clone R73), and FITC anti-rat CD45RC (clone OX-22) (BD Biosciences Pharmingen) respectively.

Cytokine measurements

Cytokines in cell culture supernatants were measured by ELISA using unlabeled capture antibodies and biotin-labeled detection antibodies, followed by horse-radish peroxidase labeled Streptavidin.[7] The concentration of each cytokine was determined by comparison to a standard curve generated using recombinant proteins. For IFN-γ, we used unlabeled polyclonal anti-IFN-γ antibody and biotin-labeled monoclonal anti-IFN-γ antibody (clone DB-1) (Biosource International, Camarillo, CA). For IL-10 ELISA we used unlabeled monoclonal anti-rat IL-10 antibody (clone A5-7) and biotin-labeled monoclonal anti-rat IL-10 antibody (clone A5-6) (BD Biosciences Pharmingen, San Diego, CA).

Cecal cytokine analysis

Cecal cytokines were measured as described previously.[17] Briefly, cecal tissues were homogenized in PBS containing a cocktail of protease inhibitors,[19] after which the homogenate was assayed for IL-1β according to the manufacturer's instructions (National Institute for Biological Standards and Controls, South Mimms, UK).

MPO assay

Homogenized ceca were assayed for MPO activity (units per gram of cecal tissue) as described previously.[17,20]

Statistical analysis

Cytokine levels are expressed as mean ± SEM in triplicate measurements. A non-paired Student t-test was used, in which a two-tailed p value of < 0.05 was considered statistically significant.

Results

Donor and recipient mesenteric lymph node cell populations before cell transfer

Unseparated mesenteric lymph node cells from *rnu/+* non-transgenic or transgenic rats were used as donor cells for adoptive transfer. The transgenic donor rats had moderate colitis as shown by histology scores of 1.5-2.0 on a 0-4 scale (data not shown). Non-transgenic donor mesenteric lymph node cells contained a higher proportion of B cells and a lower proportion of T cells compared to those from their transgenic littermates that developed colitis (table 1). Also, both the transgenic and the non-transgenic donor cell preparations contained $CD4^+$ cells that express low levels of CD45RC (table 1). B cells were the predominant cell types in mesenteric lymph node of *rnu/rnu* non-transgenic and transgenic recipients (table 1). We evaluated cell surface expression of HLA-B27 to confirm the non-transgenic or transgenic status of the donor and recipient rats.

	Donor		**Recipient**	
	Non-Transgenic n=6	Transgenic n=5	Non-Transgenic n=2	Transgenic n=2
Percent				
TCRαβ	49.4 ± 1.7	66.6 ± 1.9[a]	1.1 ± 0.1	1.9 ± 0.5
CD4	30.8 ± 1.8	51.8 ± 1.6[a]	2.3 ± 0.4	2.9 ± 0.1
$CD45RC^{low}/CD4^+$	17.1 ± 1.8	37.3 ± 4.4[a]	1.8 ± 0.1	2.5 ± 0.3
CD8	17.8 ± 0.5	13.7 ± 0.6[a]	1.4 ± 0.1	1.3 ± 0.1
Surface Ig	48.9 ± 1.4	31.7 ± 2.2[a]	91.0 ± 0.5	93.5 ± 0.5
HLA-B27	0.7 ± 0.2	97.9 ± 0.4[a]	1.1 ± 0.3	98.4 ± 0.1

Table 1. Cell populations (%) in mesenteric lymph nodes from donor (*rnu/+*) and recipient (*rnu/rnu*) HLA-B27 non-transgenic and transgenic rats before cell transfer. [a] $P < 0.005$ versus non-transgenic donor. TG, transgenic; non-TG, non-transgenic.

Transfer of mesenteric lymph node cells induces inflammation in transgenic but not in non-transgenic *rnu/rnu* recipients

Adoptive transfer of transgenic mesenteric lymph node cells into *rnu/rnu* transgenic recipients induced severe colitis, as shown in figure 1D. This colitis in characterized by massive infiltration of granulocytes and mononuclear cells of mucosa and submucosa, bowel wall thickening, crypt abscesses, crypt hyperplasia, and goblet cell depletion. *Rnu/rnu* non-transgenic recipients that received transgenic mesenteric lymph node cells remained disease-free, as shown in figure 1B. Interestingly, adoptive transfer of non-transgenic mesenteric lymph node cells into *rnu/rnu* transgenic recipients induced moderate colitis (figure 1C), whereas *rnu/rnu* non-transgenic recipients receiving non-transgenic mesenteric lymph node cells did not show any signs of intestinal inflammation (figure 1A). Histologic inflammatory scores (scale 0-4) confirmed the severe inflammation in both cecum and colon in *rnu/rnu* transgenic recipients receiving transgenic mesenteric lymph node cells, and moderate intestinal inflammation in *rnu/rnu* transgenic recipients receiving non-transgenic mesenteric lymph node cells (figure 2A-B). No disease was detected in *rnu/rnu* non-transgenic rats receiving either non-transgenic or transgenic mesenteric lymph node cells ($P < 0.005$ versus transgenic recipients) (figure 2A-B).

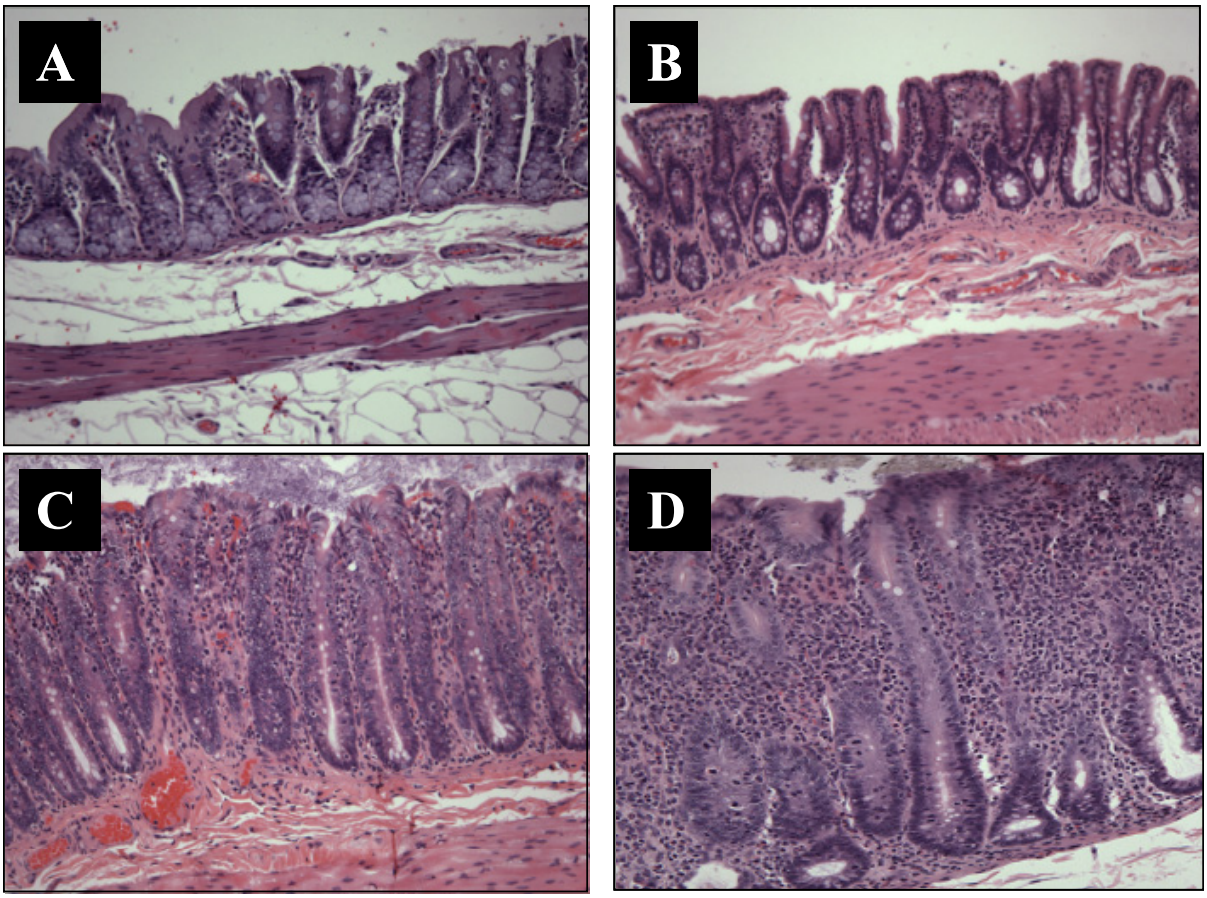

Figure 1. Cecal inflammation in recipients of mesenteric lymph node cell transfers. Representative sections of the cecum of *rnu/rnu* transgenic and non-transgenic recipient rats, eight weeks after transfer of mesenteric lymph node cells from *rnu/+* transgenic or non-transgenic donors are shown at 10X magnification. Panels represent non-transgenic donor cells transferred into non-transgenic (A) or transgenic (C) recipients, and transgenic donor cells transferred into non-transgenic (B) and transgenic (D) recipients. TG, transgenic; non-TG, non-transgenic.

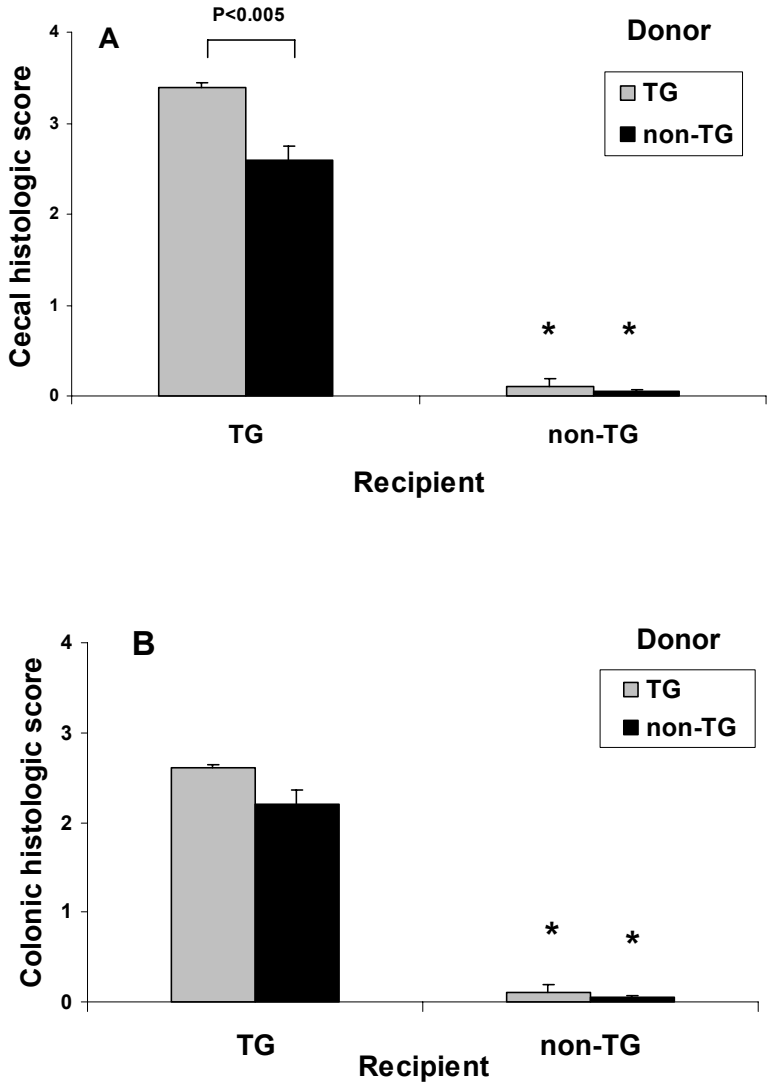

Figure 2. Cecal and colonic histologic scores for recipients of mesenteric lymph node cell transfers. Intestinal tissue was collected from transgenic and non-transgenic *rnu/rnu* recipient rats, eight weeks after transfer of mesenteric lymph node cells from *rnu/+* transgenic or non-transgenic donors. Blinded histology scores for cecal (A) and colonic (average of proximal, transverse, and distal colon) (B) tissue are shown. Values represent mean ± SEM, n=5-8 rats per group. * $P < 0.005$ for histologic scores of non-transgenic recipient tissue compared to transgenic recipient tissue. TG, transgenic; non-TG, non-transgenic.

Flow cytometric analysis of mesenteric lymph node cells in *rnu/rnu* recipient rats after adoptive transfer

Evaluation of cell subpopulations was carried out on mesenteric lymph node cells collected from all recipients, as shown in table 2. Eight weeks after mesenteric lymph node cell transfer, cells carrying the HLA-B27 molecule comprised 40% of the total mesenteric lymph node cell population in *rnu/rnu* transgenic recipients receiving non-transgenic mesenteric lymph node cells. In contrast, no HLA-B27 positive cells were detected after transfer of transgenic mesenteric lymph node cells into *rnu/rnu* non-transgenic recipients, a consistent finding observed in three independent experiments. Of interest, equivalent proportions of $CD4^+$ T cells were present in the *rnu/rnu* transgenic recipients of either transgenic or non-transgenic donor mesenteric lymph node cells. Mesenteric lymph nodes of *rnu/rnu* non-transgenic recipients of transgenic mesenteric

lymph node cells contained significantly lower proportions of T cells and higher percentages of B cells than did *rnu/rnu* non-transgenic recipients of non-transgenic mesenteric lymph node cells.

Donor **Recipient**	Transgenic Transgenic n=5	Non-Transgenic Transgenic n=8	Transgenic Non-Transgenic n=6	Non-Transgenic Non-Transgenic n=5
Total Cells				
x 10^6	52 ± 12	83 ± 21	60 ± 7	46 ± 9
Percent				
TCRαβ	33.8 ± 3.7[b]	34.2 ± 2.6[b]	5.3 ± 1.2[b]	14.9 ± 1.2
CD4	30.8 ± 3.4[b]	29.6 ± 2.5[b]	4.1 ± 0.8b	13.2 ± 1.2
CD8	1.5 ± 0.2	4.2 ± 0.6	2.8 ± 0.3	2.2 ± 0.3
Surface Ig	56.4 ± 5.4[b]	60.0 ± 3.3[b]	89.5 ± 1.0[a]	77.7 ± 2.5
HLA-B27	92.5 ± 1.6[b]	40.6 ± 4.8[b]	0.4 ± 0.2	0.5 ± 0.1

Table 2. Cell numbers and cell populations in mesenteric lymph nodes from *rnu/rnu* HLA-B27 transgenic rats after mesenteric lymph node cell transfer. Values represent mean ± SEM of percentages of the different lymphoid cell populations in mesenteric lymph node of recipients analyzed eight weeks after mesenteric lymph node cell transfer. TG, transgenic; non-TG, non-transgenic.
[a] $P < 0.01$ versus non-transgenic donor cells transferred into non-transgenic recipients.
[b] $P < 0.005$ versus non-transgenic donor cells transferred into non-transgenic recipients.

Increased cecal MPO and IL-1β in *rnu/rnu* transgenic recipients

Inflammation in diseased rats was most severe in the cecum. Therefore, we measured myeloperoxidase levels in cecal tissue as a parameter of inflammation, and more specifically, granulocyte and macrophage infiltration. *Rnu/rnu* transgenic recipients receiving transgenic mesenteric lymph node cells had higher cecal MPO levels (albeit not statistically significant) and IL-1β levels ($P < 0.005$) than *rnu/rnu* transgenic recipients of non-transgenic mesenteric lymph node cells as shown in figure 3A and 3B. However, cecal MPO and IL-1β levels were both significantly higher in transgenic recipients of non-transgenic mesenteric lymph node cells than those found in cecal tissue from *rnu/rnu* non-transgenic recipients receiving mesenteric lymph node cells from either type of donor.

Cytokine profile in mesenteric lymph node cell cultures from *rnu/rnu* transgenic recipients after mesenteric lymph node cell transfer

Previously, we have shown that $CD4^+$ T cells are critical for IFN-γ production in HLA-B27 transgenic rats, whereas B cells are required for IL-10 secretion.[7] In the present study, mesenteric lymph node cells from recipients were isolated and stimulated *in vitro* with cecal bacterial lysate. IFN-γ secretion in response to stimulation by components of cecal bacteria present in the lysate was significantly higher in *rnu/rnu* transgenic recipients compared to *rnu/rnu* non-transgenic recipients ($P < 0.005$), as shown in figure 4A. mesenteric lymph node cells collected from *rnu/rnu* transgenic recipients as early as one week after transfer of transgenic donor cells produced higher amounts of IFN-γ in response to cecal bacterial lysate compared to stimulated mesenteric lymph node cells from *rnu/rnu* non-transgenic recipients (figure 5). Interestingly, the amounts of IFN-γ detectable in the supernatants of the recipient transgenic

mesenteric lymph node cells collected from *rnu/rnu* transgenic recipients at this early time point after transfer were higher than the amounts produced by cecal bacterial lysate-stimulated mesenteric lymph node cells from *rnu/+* transgenic rats with colitis (figure 5). IL-10 produced in response to cecal bacterial lysate was significantly higher in cultures of *rnu/rnu* transgenic recipients of non-transgenic mesenteric lymph node cells compared to *rnu/rnu* transgenic recipients of transgenic mesenteric lymph node cells ($P < 0.01$, figure 4B).

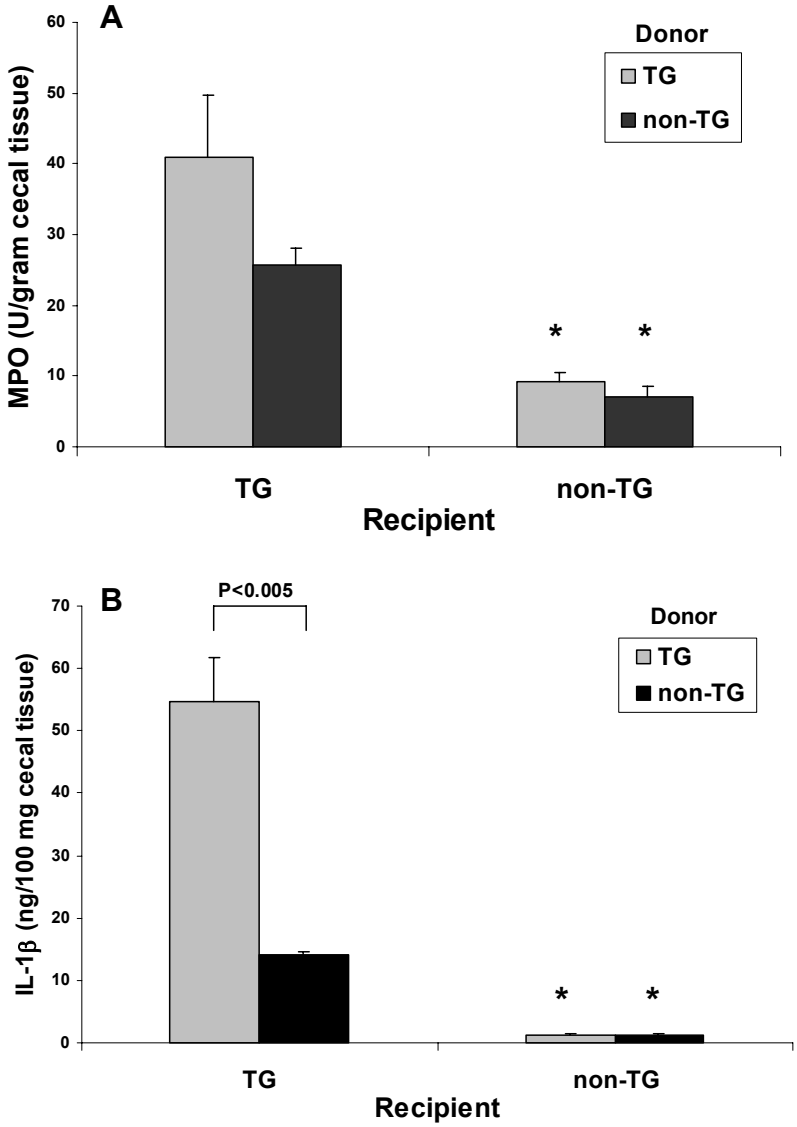

Figure 3. MPO and IL-1β in cecal tissue of cell transfer recipients. Transgenic and non-transgenic mesenteric lymph node cells from *rnu/+* rats were transferred into *rnu/rnu* transgenic and non-transgenic recipients. After eight weeks, cecal tissue was collected, homogenized, and MPO (A) and IL-1β (B) were determined in duplicate supernatants. Values represent mean ± SEM, n=5-8 rats per group. *$P < 0.005$ for cecal tissue from non-transgenic recipients compared to transgenic recipients. TG, transgenic; non-TG, non-transgenic

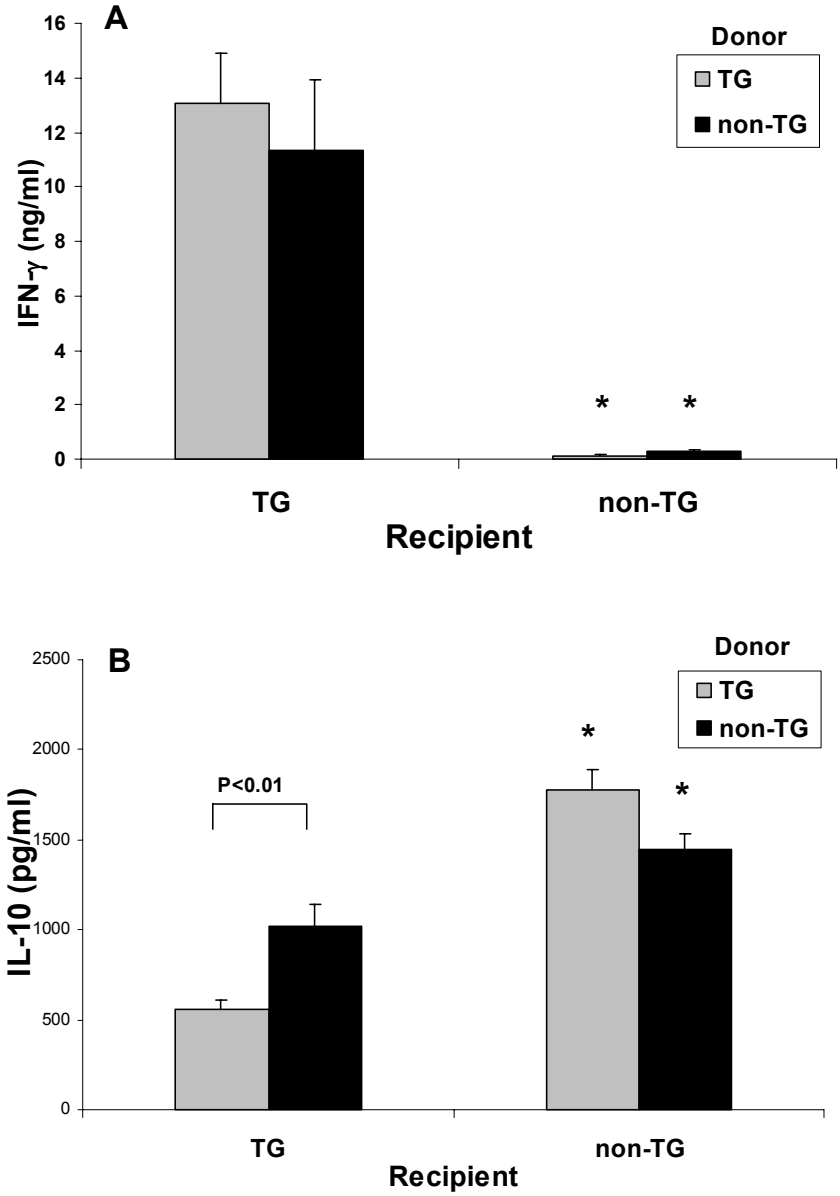

Figure 4. Cytokine production by recipient mesenteric lymph node cells after adoptive mesenteric lymph node cell transfer. Mesenteric lymph node cells were collected from transgenic and non-transgenic recipient rats, eight weeks after transfer of mesenteric lymph node cells from *rnu/+* transgenic or non-transgenic donors and cultured in the presence or absence of cecal bacterial lysate. After three days, supernatants were collected and IFN-γ stimulated by cecal bacterial lysate at 50 μg/ml (A) and IL-10 stimulated by cecal bacterial lysate at 10 μg/ml (B) were measured in triplicate supernatants by ELISA. Values represent mean ± SEM, n=5-8 rats per group. IFN-γ levels were significantly lower (*P < 0.005) and IL-10 levels were significantly higher (*P < 0.005) in supernatants of cecal bacterial lysate stimulated mesenteric lymph node from non-transgenic recipients compared to transgenic recipients. TG, transgenic; non-TG, non-transgenic.

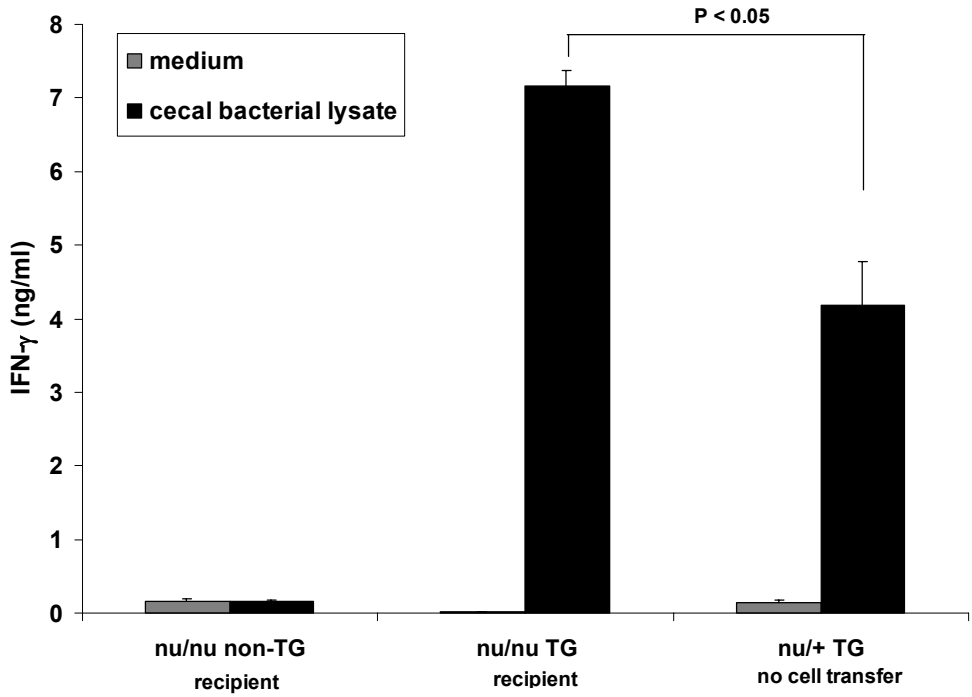

Figure 5. IFN-γ production by cecal bacterial lysate stimulated mesenteric lymph node cells. Mesenteric lymph node cells from *rnu/+* transgenic rats were transferred into *rnu/rnu* non-transgenic or *rnu/rnu* transgenic recipients. After seven days, recipient mesenteric lymph nodes were harvested, and cells were stimulated with cecal bacterial lysate at 50 μg/ml or cultured without stimulation (medium). Mesenteric lymph nodes were also obtained from *rnu/+* transgenic rats (not transplanted) and stimulated with cecal bacterial lysate or cultured with medium. Supernatants were collected after three days. IFN-γ was measured in triplicate supernatants by ELISA. Values represent mean ± SD of triplicate measurements from mesenteric lymph node cell cultures of individual animals. Results are representative of two independent experiments. TG, transgenic; non-TG, non-transgenic.

Transgenic mesenteric lymph node donor cells proliferate and accumulate in transgenic but not in non-transgenic recipients

To obtain a better understanding of the kinetics of donor cell survival and proliferation, we evaluated CFSE-labeled transgenic donor cells after transfer into either *rnu/rnu* non-transgenic or *rnu/rnu* transgenic recipients. CFSE-labeled dividing cells can be easily monitored due to the incremental reduction in CFSE fluorescence intensity that occurs with each cell division. On day two after transfer, two-fold more CFSE-labeled cells were detectable in the mesenteric lymph nodes of transgenic recipients compared to non-transgenic recipients (figures 6A-B versus 6E-F). Two populations of TCRαβ-expressing cells are present (figure 6B and 6F). One is CFSE positive, identifying donor cells, the other is CFSE-negative. The CFSE-negative cells are either donor cells that divided rapidly after transfer and thus lost detectable CFSE, or alternatively, these cells could represent the minor population of TCRαβ-expressing cells that are present in the mesenteric lymph nodes of *rnu/rnu* recipients (see table 1). At day seven, mesenteric lymph nodes of non-transgenic recipients no longer contain CFSE-labeled cells (figure 6C-D) while mesenteric lymph nodes of transgenic recipients contain CFSE-labeled TCRαβ-expressing cells that had divided, as evidenced by their reduced fluorescence intensity (figure 6G-H). It is interesting to note that the transferred cells that do not express TCRαβ are not dividing (figure 6H, lower right quadrant). We also evaluated spleen cells of the recipients to determine if donor cells were more or less abundant outside the mesenteric lymph nodes. More CFSE-labeled donor cells were consistently seen in the mesenteric lymph nodes than in the spleen of either transgenic or non-

transgenic recipients (data not shown), excluding the possibility that donor mesenteric lymph node cells home to the spleen in non-transgenic rats.

Discussion

The results of the present study show that adoptive transfer of mesenteric lymph node cells from either transgenic donor rats that develop colitis or from disease-free non-transgenic donors into athymic transgenic recipients induces inflammation in the cecum and colon. This finding implicates accessory cells that express the HLA-B27 transgene as the activators of disease-inducing T cell responses.

As demonstrated previously, T cells are required for inflammation in HLA-B27 transgenic rats, since *rnu/rnu* transgenic rats remain disease-free.[8] Moreover, $CD4^+$ T cells have been shown to mediate inflammatory responses in HLA-B27 transgenic rats,[8] and they produce large amounts of IFN-γ when stimulated with components of commensal bacteria.[7] Peptides bound in the peptide binding grooves of MHC class I molecules such as HLA-B27 are usually associated with activation of $CD8^+$, not $CD4^+$ T cells. However, it has been clearly shown that CD4, and not CD8 cells, are crucial for disease-induction in HLA-B27 transgenic rats. $CD4^+$ T cells were much more efficient than $CD8^+$ T cells in transferring disease into nude HLA-B27 rats.[8] Additionally, the minor role for $CD8^+$ T cells in disease was shown by the lack of an effect on colitis by either anti-CD8 treatment or CD8 depletion in HLA-B27 rats.[21] In our study, CD8 cells did not proliferate and the percentage of $CD8^+$ cells detected by flow cytometry after mesenteric lymph node cell transfer is low in all *rnu/rnu* recipients.

$CD4^+$ T cell subpopulations with regulatory functions have been identified. In an elegant study, Powrie et al showed that transfer of $CD4^+CD45RB^{hi}$ T cells from normal mice induced colitis in SCID mice. This inflammatory process could be prevented by co-transfer of $CD4^+$ cells that express low levels of CD45RB from normal mice.[9] The same principle was shown in athymic rats, where transfer of T cells that express high levels of the CD45 isoform, designated $CD45RC^{hi}$, mediated inflammation in several different organ systems, whereas the severity of inflammation was greatly reduced by co-transfer of a $CD4^+CD45RC^{low}$ cell population.[10] Also B cells and intraepithelial lymphocytes have been suggested as regulators of inflammation.[11,22] Taken together, analysis of these different models of colitis indicates that disease induction as well as regulation are complex processes that require interactions among a variety of different cell types.

In our study, we transferred a mixed cell population of non-transgenic or transgenic mesenteric lymph node cells into *rnu/rnu* athymic HLA-B27 transgenic rats, resulting in moderate to severe colitis in the recipients. Donor cells were unfractionated and contained both $CD45RC^{low}$ $CD4^+$ and $CD45RC^{hi}$ $CD4^+$ cells, as well as B cells and $CD8^+$ cells. We have shown previously that mesenteric lymph node-derived rat B cells, but not T cells, produce IL-10 and TGF-β in response to components of commensal bacteria.[7] Our current findings indicate that $CD45RC^{low}$ $CD4^+$cells or B cells, from either transgenic or non-transgenic donors, failed to prevent development of inflammation in *rnu/rnu* transgenic recipients. Conversely, *rnu/rnu* non-transgenic recipients that do not express HLA-B27 remain healthy, even after transfer of mesenteric lymph node cells from transgenic donors with colitis.

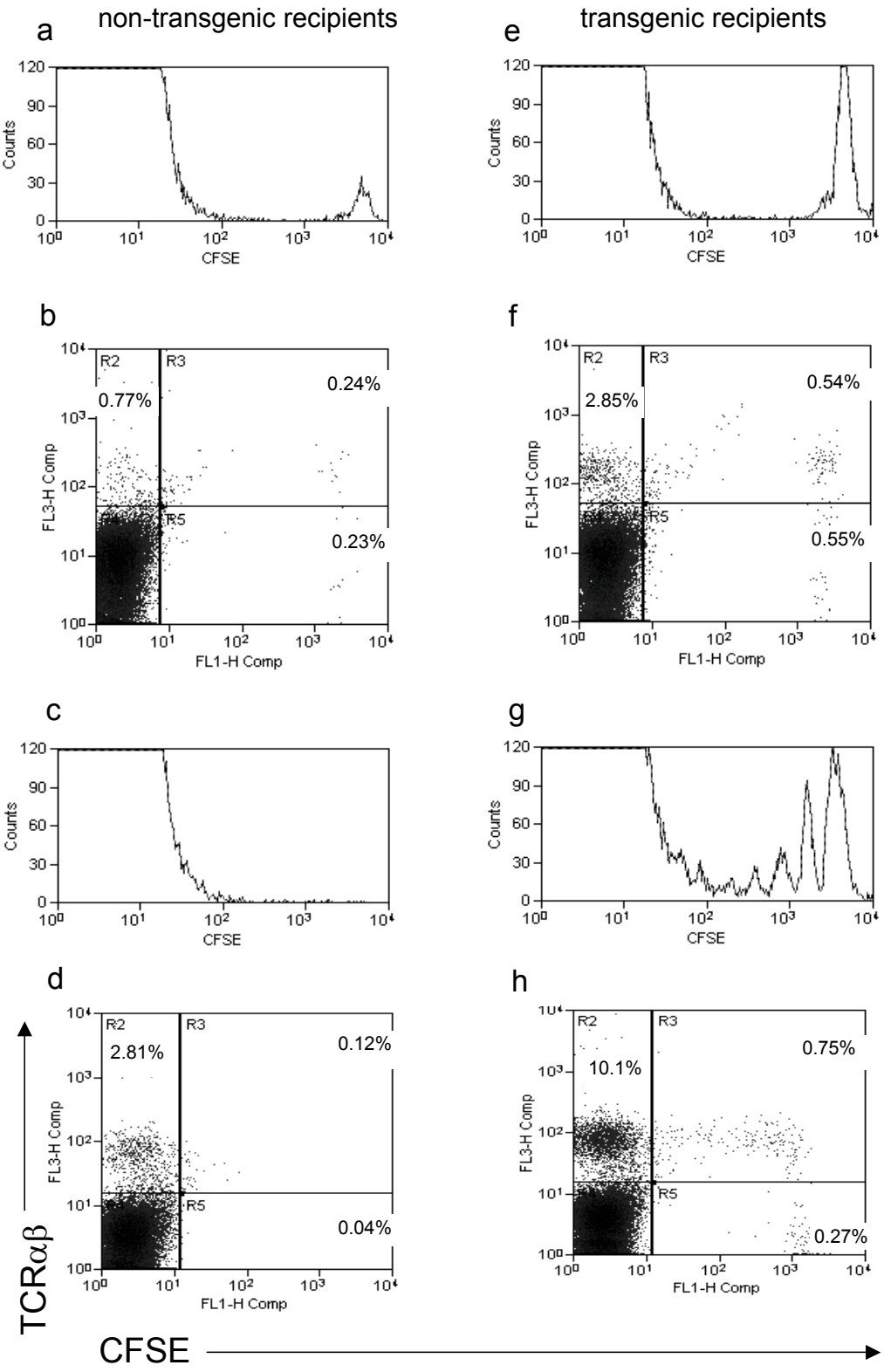

Figure 6. CFSE labeled mesenteric lymph node cells from *rnu/+* transgenic rats were transferred into *rnu/rnu* non-transgenic (A, B, C, D) or *rnu/rnu* transgenic recipients (E, F, G, H). After two (A, B, E, F) and seven days (C, D, G, H), recipient mesenteric lymph node cells were collected and analyzed by flow cytometry to enumerate CFSE-labeled cells (histograms) and cells that express TCRαβ (dot plots). Values represent percent positive cells in the labeled quadrants.

Cecal inflammation was more severe and cecal IL-1β levels were higher in *rnu/rnu* transgenic rats after transfer of transgenic mesenteric lymph node cells than non-transgenic mesenteric lymph node cells. A number of explanations can be considered, including transfer of greater numbers of T cells (table 1) as well as previously activated T cells from transgenic compared to non-transgenic donors that are capable of responding rapidly to stimulatory signals. IL-10 is an important immunoregulatory molecule.[23] For example, IL-10 is produced by regulatory T cells and is crucial for the inhibition of colitis in the SCID mouse $CD4^+CD45RB^{hi}$ cell transfer model.[24] In another study, IL-10 producing C3H/HeJBir $CD4^+$ T regulatory cells, generated *in vitro* by sequential stimulation with cecal bacterial antigens in the presence of IL-10, prevented onset of colitis in SCID recipients after co-transfer with colitis inducing T cells.[25] In HLA-B27 transgenic and non-transgenic rats, B cells are the main source of IL-10.[7] Although less IL-10 is secreted by mesenteric lymph node cells from *rnu/rnu* transgenic recipients compared to those from *rnu/rnu* non-transgenic recipients, mesenteric lymph node cells of rats from both groups were able to produce IL-10. However, the capacity of these cells to produce IL-10 was not sufficient to downregulate cecal bacterial lysate-induced IFN-γ secretion by mesenteric lymph node cells from *rnu/rnu* transgenic recipients. In preliminary experiments we have identified hyporesponsiveness of transgenic mesenteric lymph node cells to IL-10 that might also explain the inability of IL-10 to inhibit development of colitis in transgenic recipients.[26]

A major finding in our study is that accessory cells carrying the HLA-B27 molecule determine the outcome of effector T cell responses. This is clearly demonstrated by the finding that non-transgenic T cells, that do not induce disease in non-transgenic hosts, are capable of inducing colitis when transferred to *rnu/rnu* transgenic recipients. When the same cells are transferred to *rnu/rnu* non-transgenic recipients, no disease develops, indicating that the *rnu/rnu* host expressing transgenic HLA-B27 orchestrates T cell responses to commensal bacteria. The importance of HLA-B27 in the activation of T cell responses was further underlined by our observation that addition of anti-HLA-B27 antibodies to co-cultures of transgenic $CD4^+$ T cells plus cecal bacterial lysate pulsed transgenic antigen presenting cells reduced IFN-γ responses.[27]

How can we explain that accessory cells determine disease outcome, but that no disease occurs without T cells? There is growing evidence that T cells are not acting in an independent fashion, but are influenced by the innate immune system.[28] TLR are crucial for innate immune responses against microbial products.[29] These responses can skew the acquired immune system, including T cells, into regulatory or proinflammatory directions. Therefore, dendritic cells and macrophages can regulate T cell responses not only by their direct antigen presentation capacities, but also indirectly by the production of cytokines that are induced after TLR signaling.[30]

If $CD4^+$ T cells, and not $CD8^+$ T cells are required, how does the MHC class I molecule HLA-B27 induce development of inflammatory diseases? Although this question has not yet been answered definitively, a number of different mechanisms have recently been proposed. Boyle et al described several different forms of HLA-B27, such as heterodimers that lack bound peptides. These molecules can associate to form either empty or peptide-binding homodimers.[31] Support for the formation of HLA-B27 homodimers has been reported by Antoniou et al.[32] Furthermore, other studies identified human $CD4^+$ T cells that recognized unconventional forms of HLA-B27.[33] Although $CD4^+$ T cells are considered important for induction of intestinal inflammation in HLA-B27 transgenic rats, a recent publication showed that mice transgenic for a TCR that recognizes B27+ peptide have both CD4 and CD8 cells expressing the transgenic TCR that can see B27.[34] However, HLA-B27 molecules alone are not sufficient to trigger inflammation, since both germ-free HLA-B27 transgenic rats[3,6] and specific pathogen-free *rnu/rnu* transgenic rats[8] do not

develop colitis. Taken together, the combination of HLA-B27, $CD4^+$ T cells, and the presence of intestinal bacteria, is required for development of disease in HLA-B27 transgenic rats.

It is unclear what process determines the poor survival of transgenic mesenteric lymph node cells in the *rnu/rnu* non-transgenic recipients. One possibility is that, for this donor recipient combination, the donor cells proliferate only minimally, and therefore form a minor population relative to the endogenous cell population in the recipients after eight weeks. This is supported by the results of our cell transfer experiment using CFSE labeled cells. Multiple cycles of proliferation were detected in transgenic recipients but not in non-transgenic recipients on day seven after mesenteric lymph node cell transfer. Secondly, transgenic donor cells could be killed by NK cells of the non-transgenic host. Cells carrying high copy numbers of HLA-B27 have reduced levels of endogenous MHC class I molecules.[2] It is possible that this feature makes transgenic donor cells prone to NK cell-mediated lysis.[35,36] A third possibility is that the donor transgenic mesenteric lymph node cells might undergo apoptosis following transfer into *rnu/rnu* non-transgenic recipients, because their activation and subsequent survival depends on interactions with HLA-B27-expressing accessory cells. Finally, other organs such as spleen, liver or lungs might trap donor cells, thereby preventing these cells from reaching the mesenteric lymph node. Although we cannot rule out trapping of donor cells in non-lymphoid tissues, we detected a higher percentage of CFSE-labeled donor cells in mesenteric lymph nodes than in the spleen of transgenic recipients, supporting the notion that mesenteric lymph nodes constitute an important site for activation, proliferation, and survival of the transplanted cells.

Transfer of non-transgenic donor cells into transgenic recipients can potentially induce graft versus host disease, including systemic inflammatory lesions. However, we think that the disease we have documented in transgenic recipients of non-transgenic mesenteric lymph node cells cannot be explained by a graft versus host reaction. In an extensive description of graft versus host disease in the rat, no inflammation of the large intestine was observed, whereas moderate to several cecal and colonic inflammation occurred in *rnu/rnu* transgenic recipients in our study.[37] Second, the colitis that developed in transgenic recipients following transfer of either transgenic or non-transgenic cells showed minor differences in severity and were very similar in phenotype and histological features (figure 1). Thus, it is unlikely that the inflammation we observed following transfer of non-transgenic mesenteric lymph node is the result of a graft versus host reaction.

Several published reports show reduced capacity of accessory cells, such as dendritic cells, from HLA-B27 transgenic rats to activate allogenic cells *in vitro*.[38,39] The results of our study, while not directly addressing co-stimulatory activity, indicate that cells of *rnu/rnu* transgenic recipients are potent stimulators of transferred syngeneic donor mesenteric lymph node cells *in vivo*. The role of accessory cells in determining the nature of the immune response has been extensively evaluated, and the results indicate that antigen-presenting cells are crucial for both activation and also for subsequent down-regulation of immune responses.[40] The results of our *in vivo* transfer study indicate that accessory cells in *rnu/rnu* transgenic recipients may lack the capacity to inhibit the colitis-inducing abilities of $CD4^+$ T cells from donor rats that interact with HLA-B27 expressing cells.

In conclusion, we have shown that colitis develops in *rnu/rnu* transgenic rats receiving transgenic or non-transgenic mesenteric lymph node cells. In contrast, *rnu/rnu* non-transgenic rats remained disease-free after mesenteric lymph node cell transfer, indicating that the accessory cells of the nude rats that carry HLA-B27 orchestrate T cell responses to luminal commensal bacteria. The results of our investigation underscore the importance of HLA-B27 expressing accessory cells in regulating pathogenic host mucosal immune responses to commensal bacteria.

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STAT3 regulates NF-κB recruitment to the IL-12 p40 promoter in dendritic cells

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Frank Hoentjen[1,2], R. Balfour Sartor[1]
Michitaka Ozaki[3], and Christian Jobin[1]

[1]Center for Gastrointestinal Biology and Disease
University of North Carolina at Chapel Hill, USA

[2]Department of Gastroenterology
Free University Medical Center, Amsterdam, The Netherlands

[3]Department of Innovative Surgery
National Research Institute for Child Health and Development, Tokyo, Japan

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Abstract

Background and aims

IL-10$^{-/-}$ mice develop an IL-12 mediated intestinal inflammation in the absence of endogenous IL-10. The molecular mechanisms of the dysregulated IL-12 responses in IL-10$^{-/-}$ mice are poorly understood. In this study, we investigated the role of NF-κB and STAT3 in LPS-induced IL-12 p40 gene expression in BMDC isolated from wild-type and IL-10$^{-/-}$ mice.

Methods

We used LPS-stimulated BMDC from wild-type and IL-10$^{-/-}$ mice. RT-PCR and ELISA were used to determine IL-12 p40, TNF, and IL-10 expression, and we performed western blot for IkBα degradation/phosphorylation and RelA phosphorylation (S536) and STAT3 phosphorylation. Immunofluorescence for cRel and FLAG detection was carried out and adenoviral gene transfer (Ad5IkBAA, Ad5kBLUC, Ad5STAT3C) and chromatin immunoprecipitation (ChIP) for NF-κB binding to the IL-12 p40 promoter were used.

Results

We report higher IL-12 p40 mRNA accumulation and protein secretion in LPS-stimulated BMDC isolated from IL-10$^{-/-}$ compared to wild-type mice. LPS-induced NF-κB signaling is similar in IL-10$^{-/-}$ and wild-type BMDC as measured by IκBα phosphorylation and degradation, RelA phosphorylation and nuclear translocation, and NF-κB transcriptional activity, with no downregulatory effects of exogenous IL-10. ChIP demonstrated enhanced NF-κB (cRel, RelA) binding to the IL-12 p40 promoter in IL-10$^{-/-}$ but not wild-type BMDC. Interestingly, LPS induced STAT3 phosphorylation in wild-type but not IL-10$^{-/-}$ BMDC, a process blocked by IL-10 receptor blocking antibody. Adenoviral gene delivery of a constitutively active STAT3 but not control GFP virus blocked LPS-induced IL-12 p40 gene expression, and cRel recruitment to the IL-12 p40 promoter.

Conclusions

Dysregulated LPS-induced IL-12 p40 gene expression in IL-10$^{-/-}$ mice is due to enhanced NF-κB recruitment to the IL-12 p40 promoter in the absence of activated STAT3.

Introduction

IL-10$^{-/-}$ mice develop spontaneous T_H1 mediated colitis when housed under specific pathogen-free conditions.[1-3] In this experimental model, enhanced IL-12 p40 production by immune cells represents a key feature of intestinal inflammation, as demonstrated by the prevention and partial treatment of colitis by anti-IL-12 antibodies.[4,5] This suggests that in absence of IL-10, the host mounts a dysregulated innate response to the commensal intestinal microflora. Dendritic cells are at the interface of innate and adaptive immunity by virtue of their ability to secrete various cytokines including TNFα, IL-12 p40 and IL-23.[6,7] For instance, IL-12 p40 producing dendritic cells skewed T cell differentiation towards a T_H1 profile, a hallmark in the IL-10$^{-/-}$ experimental mouse model.[8,9] However, the molecular mechanisms of dysregulated IL-12 p40 gene expression in IL-10$^{-/-}$ mice following specific pathogen-free transfer are still poorly understood.

IL-10 is a potent immunoregulatory cytokine with numerous effects, such as downregulation of proinflammatory cytokines, chemokines, and costimulatory molecules.[10] Several mechanisms have been proposed for the IL-10 mediated inhibition of LPS-induced proinflammatory gene expression, including activation of the heme oxygenase/carbon monoxide pathway,[11] inhibition of the NF-κB pathway[12-14] and MAP kinase activity,[15] mRNA stability,[16] STAT3 activation,[17] and induction of Bcl-3.[18] However, the molecular mechanisms for dysregulated host innate responses in the IL-10$^{-/-}$ mouse model are still unknown. IL-10 mediates its inhibitory effects through binding to its receptor complex, which induces activation of the cytoplasmic receptor associated JAK1 and Tyk2.[10] This is followed by STAT3 phosphorylation, homodimerization, and translocation to the nucleus where it binds to STAT-binding elements in the promoters of various IL-10 inducible genes, including SOCS3 and Bcl-3.[10,18] The pivotal role of STAT3 in maintaining host homeostasis is clearly demonstrated by studies using genetic deletion. For example, STAT3 deletion is embryogenic lethal[19] and myeloid cell-specific STAT3 deficient mice develop severe enterocolitis.[20] STAT3 deletion in bone marrow cells leads to overly activated innate immune responses[21] and interferes with the adaptive immune system by inhibiting the induction of antigen-specific T cell tolerance.[22] Moreover, STAT3 gene inactivation leads to an aggressive and fatal form of enterocolitis, mediated by IL-12.[23] These data highlight the pivotal role of STAT3 in controlling innate immunity. The absence of endogenous IL-10 in IL-10$^{-/-}$ mice provides a powerful means to investigate the immunoregulatory mechanisms of this cytokine. To date, the intracellular mechanisms of dysregulated IL-12 responses in IL-10$^{-/-}$ mice have not been revealed. In this study, we demonstrate that the increased IL-12 p40 gene expression in IL-10$^{-/-}$ mice is due to enhanced NF-κB recruitment to the gene promoter caused by defective STAT3 activation. This data indicates that STAT3 plays a critical role in the resolution of LPS-induced proinflammatory gene expression and may represent a potential target for the treatment of IBD.

Methods

Cell isolation and stimulation

Wild-type and IL-10$^{-/-}$ mice (129 SvEv background) between 6 and 10 weeks of age were used to isolate bone marrow cells from femur and tibia. Red blood cells were lysed using red blood cells lysing buffer (Sigma, St. Louis, MO), and cells were cultured in 24-well low adherence plates (Costar, Corning, NY), in complete medium containing RPMI 1640 plus 10%

heat inactivated fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, 5x10^{-5} M 2-mecaptoethanol, and 50 μg/ml gentamicin in the presence of recombinant murine GM-CSF and IL-4 (both 10 ng/ml, Peprotech, Rockyhill, NJ). Floating cells were gently removed and medium was refreshed at day three, and cells were collected at day six. The cells were then washed twice and incubated overnight in regular medium without IL-4/GM-CSF. Flow cytometry analysis demonstrated a homogenous cell population with >85% CD11c^{+} cells, and <2% T cells. Additionally, MHC class II, CD80, CD86, and OX40-L cell surface expression was similar between wild-type and IL-10$^{-/-}$ BMDC, ruling against a possible phenotypic difference between the cells (data not shown). For cell stimulation, 1x10^{5} cells (cytokine measurement) or 2-4x10^{6} cells (proteins, RNA) were plated in a 96-well or 12-well plate, respectively. The cells were then stimulated with LPS (5 μg/ml, serotype 055:B5, Sigma, St. Louis, MO) in the presence or absence of recombinant murine IL-10 (12h preincubation, 10 ng/ml, Peprotech, Rockyhill, NJ).

Reagents

Purified rat anti-mouse IL-10 receptor antibody and purified rat IgG isotype control antibody were purchased from BD Pharmingen (San Diego, CA). The JAK2 inhibitor AG490 was purchased from Upstate Biotechnology (Lake Placid, NY).

Adenoviral vectors and viral infections

The constitutively active STAT3 adenovirus (Ad5STAT3C) was engineered by substituting the cysteine residues for A661 and N663, allowing STAT3 dimerization and activation without phosphorylation at Y705.[24] The STAT3C contained an extra 24 base pair DNA nucleotides encoding for the flag peptide (DYLDDDDL). The NF-κB super-repressor (Ad5IκBAA)[25] and the κB-luciferase adenoviral vector (Ad5κBLUC)[26] have been characterized and described previously. The Ad5GFP virus containing the green fluorescent protein was used as a control viral vector throughout the study.[27] The optimal multiplicity of infection for maximal infection rate was determined by flow cytometry of Ad5GFP infected BMDC, showing an infection rate of more than 80 % at an multiplicity of infection of 50 after two days of infection. Viability of adenoviral infected BMDC was comparable to uninfected cells as measured by 7-amino-actinomycin D labeling and flow cytometry analysis.

Western immunoblots

BMDC were stimulated with LPS (5 μg/ml) at various time points in 12-well plates (Costar, Corning, NY), collected, lysed in 1x Laemmli buffer and the protein concentration was measured using Bio-rad quantification assay (Bio-Rad Laboratories, Hercules, CA). Protein extracts (15 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Antibodies against phospho-IκB, phospho-RelA, phospho-p38, p38, phospho-STAT3, STAT3 (Cell Signaling, Beverly, MA), FLAG (Sigma), and IκB, RelA, and cRel (Santa Cruz Biotechnology, Santa Cruz, CA) were all used at a 1:1000 dilution. The specific immunoreactive proteins were detected using the enhanced chemo luminescence kit (ECL, Perkin Elmer), as described previously.[28]

RNA extraction and RT-PCR analysis

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA), reverse transcribed (1 μg RNA), and amplified as previously described.[28] The PCR products (7 μl) were subjected to electrophoresis on 2% agarose gels containing gel Star fluorescent dye (FMC, Philadelphia, PA).

Fluorescence staining was captured using an Alpha Imager 2000 (AlphaInnotech, San Leandro, CA). Sequences of primers used are described in Table 1.

Primer		**Sequence**	**Size** (bp)
IL-12 p40	Sense	5'-GGAAGCACGGCAGCAGAATA-3'	180
	Antisense	5'-AACTTGAGGGAGAAGTAGGAATGG-3'	
IL-10	Sense	5'-CTCTTACTGACTGGCATGAGGATC -3'	475
	Antisense	5'-CTATGCAGTTGATGAAGATGTCAAATT-3'	
TNFα	Sense	5'-ATGAGCACAGAAAGCATGATC-3'	175
	Antisense	5'-TACAGGCTTGTCACTCGAATT-3'	

Table 1. Various sequences of primers used for RT-PCR analysis.

Immunofluorescence

For FLAG expression, wild-type and IL-10$^{-/-}$ BMDC were infected for 48h with Ad5STAT3C or left uninfected. For cRel nuclear translocation, cells were stimulated with LPS (5 µg/ml) for 30 min. Subsequently, cells were fixed with 100% ice-cold methanol and permeabilized with 0.3% saponin for 10 minutes. Cells were blocked with 10% non-immune goat serum (NGS; Sigma, St-Louis, MO) for 30 min, then probed with rabbit anti-cRel antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in 10% NGS for 30 min, followed by rhodamine isothiocyanate-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in 10% NGS for 30 min. FLAG-expression was detected using mouse anti-FLAG Ab (Sigma, St. Louis, MO), 1:250 diluted in 10% NGS for 30 min, followed by a FITC-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in 10% NGS for 30 min.

Chromatin immunoprecipitation analysis

Wild-type and IL-10$^{-/-}$ BMDC were stimulated with LPS (5µg/ml) for zero, four, and eight hours, and ChIP assays were performed according to the Upstate protocol. Briefly, proteins/DNA were cross linked with 1% formaldehyde for 10 min and then cross linking was blocked with 125 mM glycine. Cells were then lysed in L1 lysis buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40, and 10% glycerol) supplemented with protease inhibitors, and chromatin was sheared by sonication (three times for 35 s, 1.0 sec on, 0.8 sec off). The extracts were pre-cleared for one hour with salmon sperm-saturated protein A/G-agarose (Upstate Biotechnology, Lake Placid, NY). Immunoprecipitation were carried out overnight at 4°C using 3 µg of anti-RelA or cRel Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were collected with salmon sperm-saturated protein A/G-agarose for two hours and washed twice in high salt buffer (20 mM Tris, pH 8.0, 0.1% SDS, 0.5 M NaCl, 1% Nonidet P-40, 2 mM EDTA) followed by two washes with no salt buffer (TE 1x), and two washes in 0.5M LiCL buffer. Samples were rotated for five min at 4°C in between every washing step. Immune complexes were extracted two times with 250 µl of freshly prepared extraction buffer (1% SDS, 0.1 M $NaHCO_2$). DNA cross-links were reverted by heating for eight hours at 65°C. After proteinase K (100 µg for one pour at 45°C)

digestion, DNA was extracted with phenol/chloroform and precipitated in ethanol. DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA (2 µl, input control) and immunoprecipitated DNA (2 µl) using IL-12 p40 promoter-specific primers as described.[29] The PCR products (8 µl) were subjected to electrophoresis on 2% agarose gels containing gel Star fluorescent dye (FMC, Philadelphia, PA). Fluorescent staining was captured using an AlphaImager 2000 (AlphaInnotech, San Leandro, CA).

Cytokine measurement

Cells were stimulated for 7-24 hours with LPS (5 µg/ml) and supernatants were collected and cytokine levels were measured using commercially available kits specific for TNFα (R&D Systems, Minneapolis, MN), and IL-12 p40 (Pharmingen/BD Biosciences), according to the manufacturers instructions. Cytokine levels were determined in triplicate culture supernatants in each separate experiment.

Statistical analysis

Statistical significance was evaluated by the two-tailed Student's *t*-test for paired data. A *P*-value <0.05 was considered statistically significant.

Results

Enhanced IL-12 p40 and TNFα gene expression in LPS-stimulated IL-10$^{-/-}$ BMDC

Since IL-10$^{-/-}$ mice develop spontaneous colitis accompanied by increased IL-12 p40 production when housed under specific pathogen-free conditions,[1,5] we first sought to evaluate the profile of T_H1 cytokine production in BMDC isolated from both IL-10$^{-/-}$ and wild-type mice. As seen in figure 1A-B, LPS induced higher IL-12 p40 and TNFα secretion (approximately 2 fold) in LPS-stimulated IL-10$^{-/-}$ compared to wild-type BMDC. Similarly, IL-12 p40 mRNA accumulation was higher in LPS-stimulated IL-10$^{-/-}$ compared to wild-type BMDC (figure 1C). This indicates that lack of endogenous IL-10 is associated with enhanced IL-12 p40 gene expression in BMDC. Interestingly, LPS induced IL-10 mRNA accumulation (figure 1C) in wild-type BMDC. As expected, IL-10 mRNA remained undetectable in IL-10$^{-/-}$ BMDC (figure 1C). Addition of exogenous IL-10 efficiently downregulated LPS-induced IL-12 p40 mRNA accumulation and TNFα protein secretion (figure 1A, B and C). This effect was blocked by IL-10 receptor blockade, but not by the isotype control IgG Ab (figure 1C). This clearly indicates that lack of IL-10-mediated signaling leads to dysregulated IL-12 p40 and TNFα gene expression.

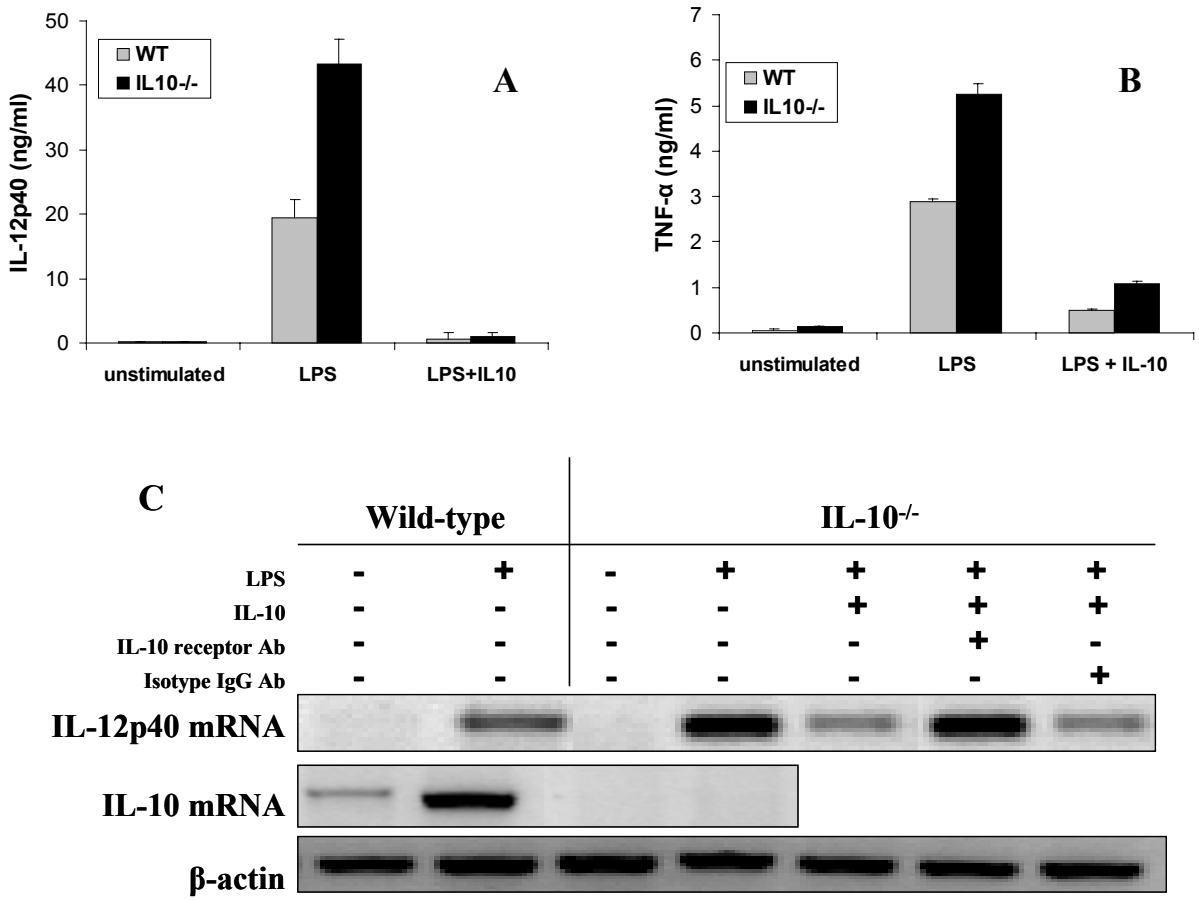

Figure 1. IL-10 mediated inhibition of increased IL-12 p40 and TNFα mRNA accumulation and protein secretion in BMDC. BMDC from wild-type and IL-10$^{-/-}$ mice were stimulated with LPS (5 μg/ml) in the presence or absence of IL-10 (10 ng/ml). (A) IL-12 p40 secretion at 24h, (B) TNFα secretion at 24h as measured in triplicate supernatants by ELISA, data presented as mean ± standard deviation. (C) IL-12 p40 and IL-10 mRNA levels as determined by RT-PCR in RNA isolated from 12h LPS-stimulated BMDC. In some samples, IL-10 receptor antibody or isotype control IgG (30 μg/ml) was used for blockade of the IL-10 receptor. The results are representative of 3 independent experiments.

IL-12 p40 secretion in IL-10-/- BMDC is NF-κB mediated

LPS signals to TLR-4 to activate numerous signaling cascades including the NF-κB pathway and its subunits. For example, cRel is important for LPS-induced IL-12 p40 transcription in macrophages and in BMDC-derived from cRel$^{-/-}$p50$^{-/-}$ mice.[30,31] However, this subunit is not required for IL-12 p40 gene expression in splenic dendritic cells.[32] We next determined the role and function of cRel in driving LPS-induced IL-12 p40 expression in IL-10$^{-/-}$ BMDC.

Immunofluorescence analysis demonstrated that cRel distribution is mainly cytoplasmic in unstimulated BMDC but clearly translocates to the nucleus in LPS-stimulated cells (figure 2A). To investigate the role of NF-κB in LPS-induced IL-12 p40 gene expression, we then utilized adenoviral vector mediated gene delivery to selectively block this signaling pathway using the IκB super-repressor (Ad5IκBAA). As seen in figure 2, LPS-induced IL-12 p40 mRNA accumulation (B) and protein secretion (C) was blocked in Ad5IκBAA- but not control Ad5GFP-infected IL-10$^{-/-}$ BMDC. Therefore, we conclude that NF-κB activation is essential for LPS-induced IL-12 p40 gene expression in IL-10$^{-/-}$ BMDC.

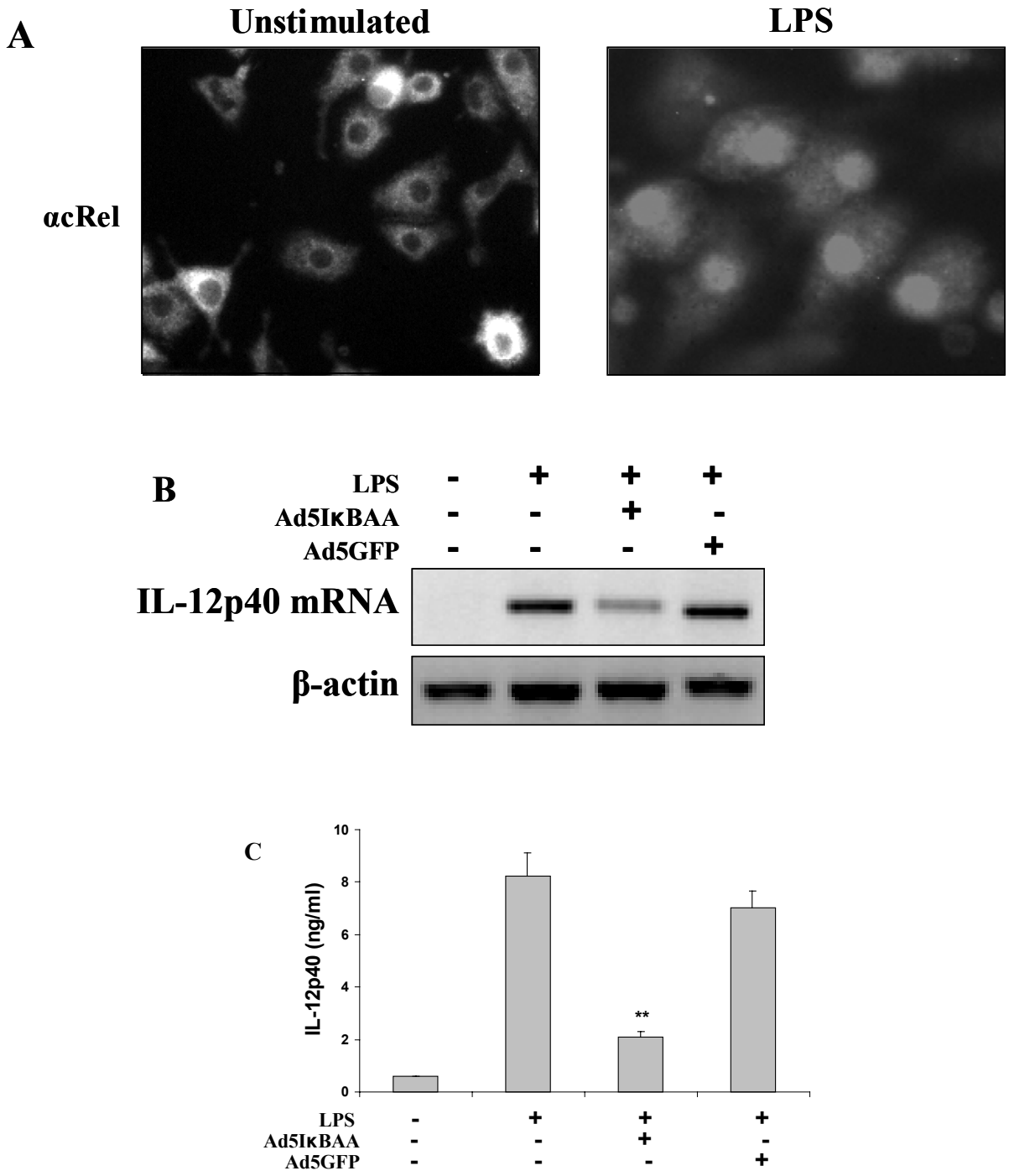

Figure 2. IL-12 p40 mRNA accumulation and protein secretion in LPS-stimulated BMDC is NF-κB dependent. (A) IL-10$^{-/-}$ BMDC were stimulated with LPS (5 μg/ml) for 30 min, and immunofluorescence detecting cRel was performed as described in the materials and methods section. (B) BMDC from IL-10$^{-/-}$ mice were infected with the IκB super repressor (Ad5IκBAA) or Ad5GFP (control) and subsequently stimulated with LPS (5 μg/ml). Cells were collected, RNA isolated, and IL-12 p40 mRNA was determined by RT-PCR. (C) IL-12 p40 secretion by Ad5IκBAA- and Ad5GFP-infected IL-10$^{-/-}$ BMDC, stimulated by LPS (5 μg/ml) for seven hours, as measured in triplicate supernatants by ELISA and presented as mean ± standard deviation. All results are representative of three independent experiments. **P<0.01.

IL-10 has been identified as a negative regulator of NF-κB activities in different cell systems.[12-14] Thus, we hypothesized that in the absence of endogenous IL-10, NF-κB signaling is enhanced and leads to increased LPS-induced IL-12 p40 gene induction. To test this hypothesis, we compared NF-κB signaling between wild-type and IL-10$^{-/-}$ BMDC. As shown in figure 3A, LPS-induced IκB degradation and phosphorylation were similar between wild-type and IL-10$^{-/-}$ BMDC. Interestingly, although exogenous IL-10 blocked LPS-induced IL-12 p40 gene expression, no effect was noticed on IκB degradation or phosphorylation (figure 3A). We recently showed that LPS-induced RelA phosphorylation at serine residue 536 is critical for IL-6 gene expression.[26] Similarly, LPS-induced RelA S536 phosphorylation is comparable between wild-type and IL-10$^{-/-}$ BMDC, and is not inhibited by exogenous IL-10 (figure 3A). Additionally, LPS-induced κB-luciferase activity is similar between wild-type and IL-10$^{-/-}$ BMDC (data not shown). In summary, activation of the proximal NF-κB signaling pathway is similar in wild-type and IL-10$^{-/-}$ BMDC stimulated with LPS.

TLR-4 activates numerous signaling cascades including the MAP pathways, which also contributes to the regulation of down-stream gene targets.[33] Since IL-10 modulates p38-activity that impacted gene expression,[11,15] we investigated whether other TLR-4 mediated p38 and JNK signaling events are affected in IL-10$^{-/-}$ cells. Therefore, we evaluated phosphorylation of the MAPK p38 and JNK in LPS-stimulated BMDC. As seen in figure 3B, p38 and JNK phosphorylation is induced in LPS-stimulated cells with no difference between wild-type and IL-10$^{-/-}$ BMDC. IL-10 pre-incubation failed to block LPS-induced p38 and JNK phosphorylation in BMDC, showing that this immunosuppressive cytokine is not targeting these MAPK. However, blocking p38 activity with SB203580 slightly impaired LPS-induced IL-12 p40 secretion (data not shown). Together, these findings suggest that although the MAPK pathway may participate with NF-κB in regulating IL-12 p40 gene expression, lack of endogenous IL-10 does not lead to dysregulated MAPK signaling. Additionally, although NF-κB signaling is critical for LPS–induced IL-12 p40 gene expression, enhanced IL-12 p40 gene expression is not accompanied by increased proximal NF-κB signaling in IL-10$^{-/-}$ BMDC.

Enhanced recruitment of cRel to the IL-12 p40 promoter in IL-10$^{-/-}$ BMDC

The recruitment of transcription factors as well as duration of binding to various gene promoters profoundly affects transcriptional activity.[34] cRel played a pivotal role in LPS induced IL-12 p40 transcription in macrophages[31] and in BMDC derived from cRel$^{-/-}$p50$^{-/-}$ mice,[30] but is dispensable in spleen-derived dendritic cells.[32] Since we observed enhanced cRel nuclear translocation (figure 2A) in LPS-stimulated BMDC, we next compared cRel recruitment to the IL-12 p40 gene promoter in wild-type and IL-10$^{-/-}$ BMDC. As seen in figure 3C, cRel is strongly recruited to the IL-12 p40 promoter in LPS-stimulated IL-10$^{-/-}$ BMDC whereas minimal loading is observed in wild-type cells. Moreover, cRel is still loaded on the IL-12 p40 promoter eight hours after LPS stimulation whereas no such recruitment is observed in wild-type BMDC (figure 3C). Interestingly, the NF-κB transcriptional subunit RelA is also strongly recruited to the IL-12 p40 gene promoter in LPS-stimulated IL-10$^{-/-}$ BMDC (figure 3C, middle panel). This suggests that enhanced IL-12 p40 gene expression in the absence of endogenous IL-10 is likely due to both enhanced binding of NF-κB (cRel/RelA) to the IL-12 p40 promoter as well as a defect in terminating gene expression, rather than increased proximal NF-κB signaling.

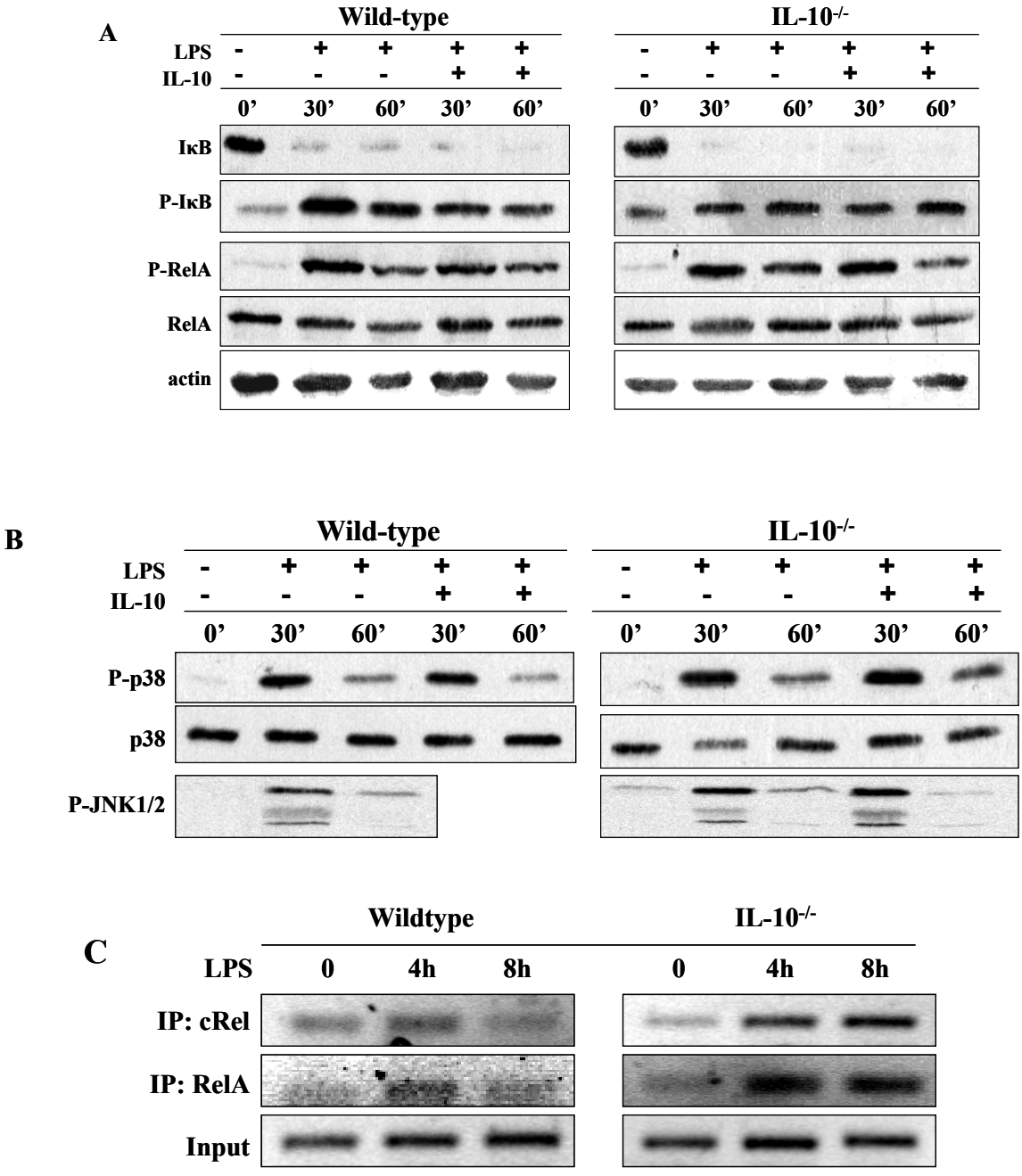

Figure 3. NF-κB signaling and MAP kinase activation in LPS-stimulated BMDC from wild-type and IL-10$^{-/-}$ mice. Wild-type and IL-10$^{-/-}$ BMDC were stimulated with LPS (5 μg/ml) in the presence or absence of IL-10 (10 ng/ml), harvested at 0, 30 and 60 min, and western blot analysis was performed for (A) phospho-IκB and IκB, and phospho-RelA and RelA, and (B) phospho-p38, p38, and phospho-JNK. The results are representative of four independent experiments. (C) wild-type and IL-10$^{-/-}$ BMDC were stimulated with LPS (5 μg/ml) for zero, four, and eight hours. ChIP analysis was performed as described in the materials and methods section. Briefly, DNA was immunoprecipitated with cRel or RelA antibody, and PCR was performed with primers specific for the IL-12 p40 gene promoter. Input samples show equal loading. Results shown are representative of three independent experiments.

STAT3 is activated by LPS-induced IL-10 in wild-type but not in IL-10$^{-/-}$ BMDC

The lack of suppressive effect of IL-10 on LPS-mediated proximal NF-κB signaling, in conjunction with a similar activation profile for various signaling events, suggests that defective negative signaling pathways may be associated with enhanced IL-12 p40 gene expression. The STAT3 pathway is required for IL-10 mediated down-regulation of LPS-induced gene expression in monocytes.[35,36] Thus, we investigated STAT3 phosphorylation in LPS stimulated wild-type and IL-10$^{-/-}$ BMDC. Interestingly, STAT3 is clearly phosphorylated between 4 and 14h in LPS-stimulated wild-type, but not in IL-10$^{-/-}$ BMDC (figure 4A). The absence of phospho-STAT3 in LPS-stimulated IL-10$^{-/-}$ BMDC is not due to an absence of STAT3 protein, as shown in figure 4A, right panel. Moreover, LPS-induced STAT3 phosphorylation in wild-type BMDC is blocked by IL-10 receptor Ab but not by control IgG Ab (figure 4B). This suggests that STAT3 activation is likely due to LPS-induced IL-10 secretion in wild-type BMDC.

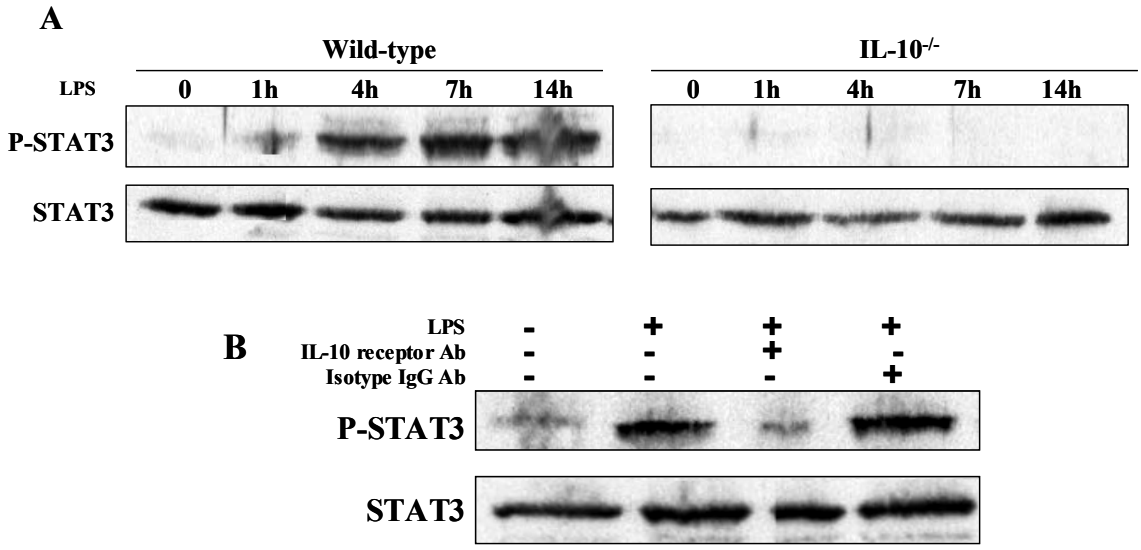

Figure 4. IL-10 dependent LPS-induced STAT3 activation in wild-type but not IL-10$^{-/-}$ BMDC. BMDC from wild-type and IL-10$^{-/-}$ mice were stimulated with LPS (5 μg/ml) in the presence or absence of IL-10 receptor antibody (30 μg/ml) or an isotype control Ab (30 μg/ml). Subsequently, cells were harvested at various time points and western blot analysis was performed for (A) phospho-STAT3 and STAT3, (B) phospho-STAT3 and STAT3 with or without IL-10 receptor blockade. The results are representative of three independent experiments.

STAT3 overexpression reduced cRel recruitment to the IL-12 p40 gene promoter in IL-10$^{-/-}$ BMDC

To investigate the impact of STAT3 on LPS-induced IL-12 p40 gene expression in IL-10$^{-/-}$ BMDC, we artificially activated this pathway by delivering a constitutively active STAT3 through adenoviral vector gene delivery (Ad5STAT3C). This adenoviral vector has been shown to trigger STAT3 signaling independently of an exogenous ligand.[24] As seen in figure 5A, FLAG-tagged STAT3C is highly expressed in Ad5STAT3C infected BMDC compared to uninfected or control Ad5GFP-infected cells. Moreover, immunofluorescence staining clearly demonstrated STAT3C expression in more than 75% of Ad5STAT3C-infected, but not in uninfected cells (figure 5B). The pattern of staining revealed a partial nuclear STAT3 localization in infected cells (figure 5B).

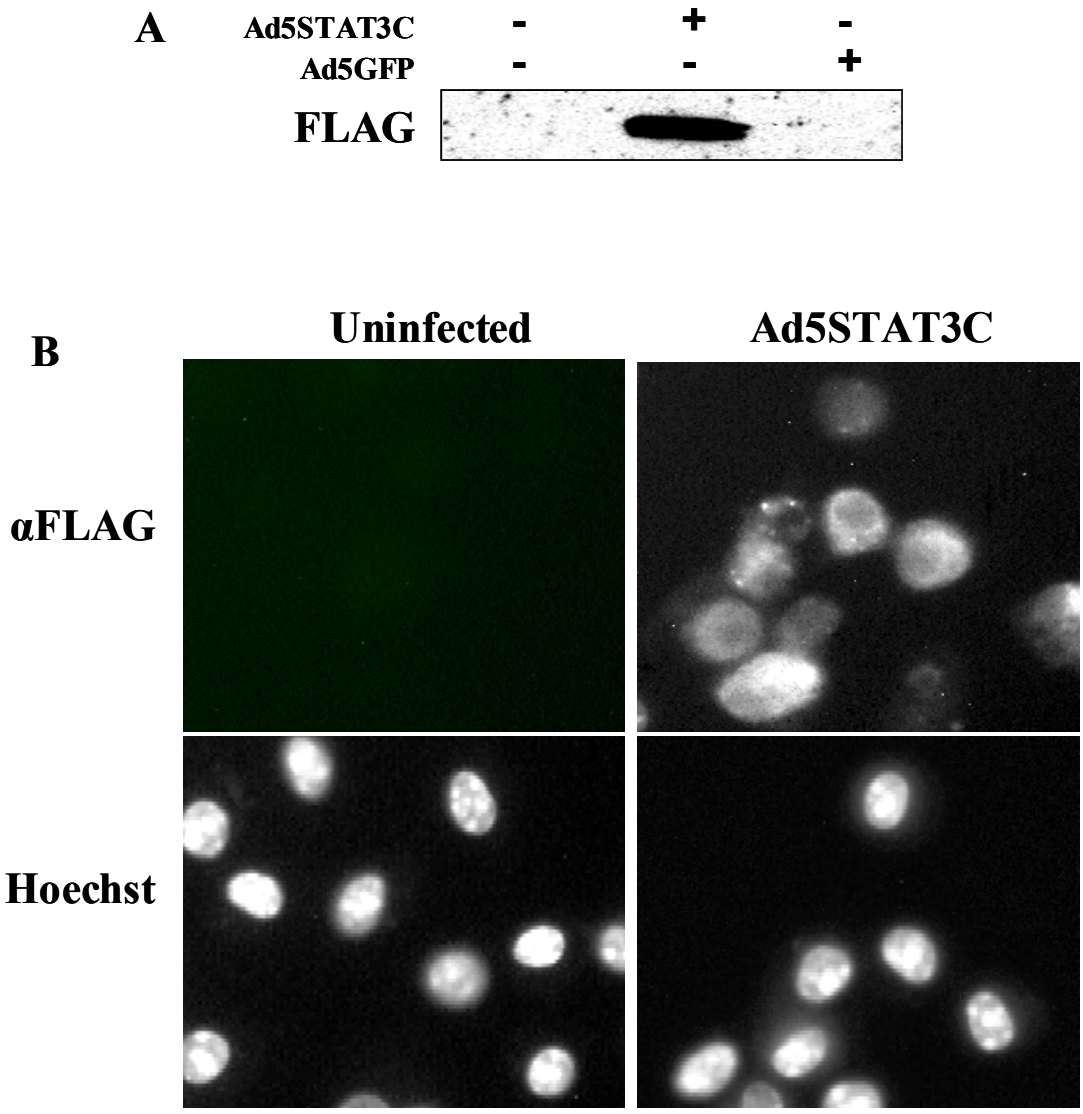

Figure 5. Overexpression of constitutively active STAT3 in IL-10$^{-/-}$ BMDC. IL-10$^{-/-}$ BMDC were infected with the constitutively active, FLAG-tagged STAT3 adenovirus (Ad5STAT3C), or Ad5GFP as a negative control. (A) After infection, BMDC were collected and protein extracts from these cells were subjected to western blot analysis using FLAG antibody. (B) After infection, cells were fixed with 100% methanol and FLAG expression was detected by immunofluorescence using FLAG antibody, followed by FITC-conjugated goat anti-mouse IgG antibody. The results are representative of three independent experiments.

Interestingly, LPS-induced IL-12 p40 mRNA accumulation is strongly blocked in Ad5STAT3C- but not in Ad5GFP-infected IL-10$^{-/-}$ BMDC (figure 6A). Additionally, LPS-induced TNFα mRNA accumulation was unaffected in Ad5STAT3C-infected cells, suggesting a selective effect of the STAT3 signaling pathway on IL-12 p40 gene expression. Similarly, Ad5STAT3C but not control Ad5GFP strongly prevented LPS-induced IL-12 p40 secretion in BMDC (figure 6B). Conversely, we used the selective JAK2 inhibitor AG490 to inhibit the JAK/STAT pathway in wild-type BMDC. This blockade increased IL-12 p40 secretion by 64 ± 2% ($P < 0.01$), confirming the importance of STAT3 in regulating IL-12 p40 responses (data not shown).

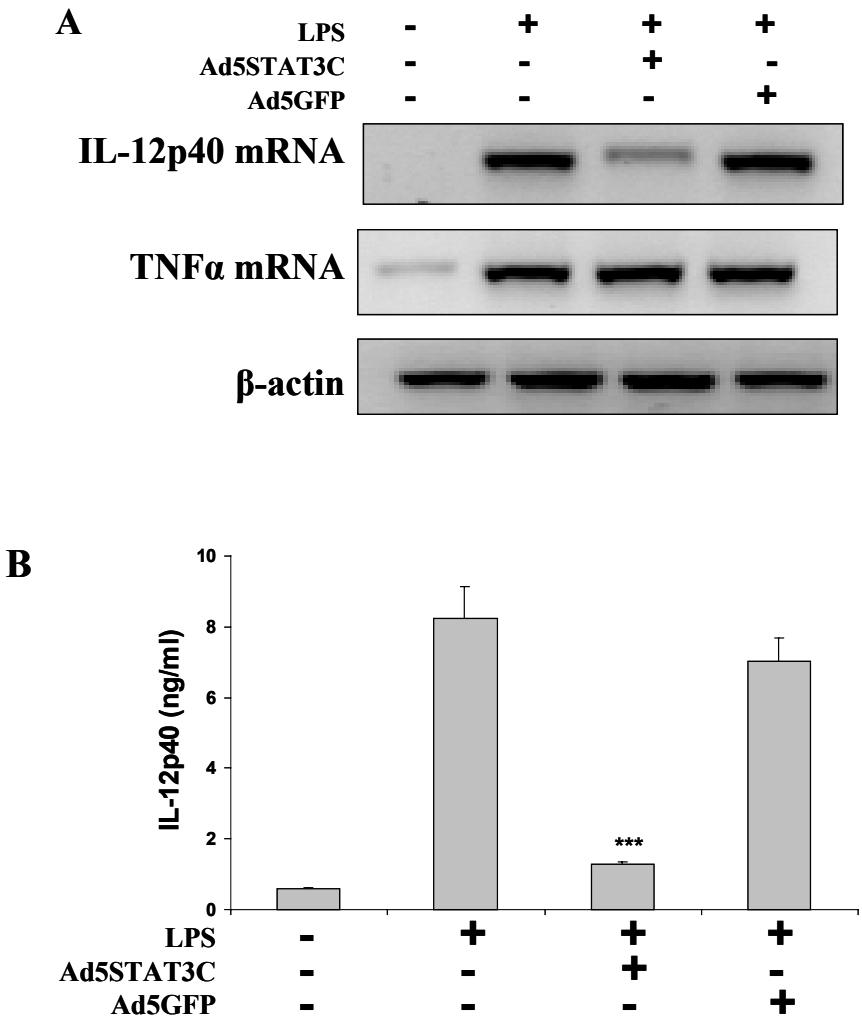

Figure 6. Adenoviral delivery of constitutively active STAT3 in IL-10$^{-/-}$ BMDC inhibits IL-12 p40 mRNA accumulation and protein secretion. IL-10$^{-/-}$ BMDC were infected with the constitutively active STAT3 adenovirus (Ad5STAT3C) or the control Ad5GFP, and subsequently stimulated with LPS (5 μg/ml). (A) Cells were collected at four hours, RNA was isolated, and IL-12 p40 and TNFα mRNA accumulation was analyzed by RT-PCR. (B) BMDC were infected as described above, stimulated with LPS (5 μg/ml) for seven hours, and IL-12 p40 secretion was measured by ELISA in triplicate supernatants and presented as mean ± standard deviation. *** P<0.005. Results are representative of two independent experiments.

We next investigated whether STAT3 interferes with LPS-induced NF-κB signaling and thus IL-12 p40 gene expression. LPS-induced IκB degradation and RelA phosphorylation were not blocked in Ad5STAT3C infected cells (figure 7A). This suggests that STAT3 blocks LPS-induced IL-12 p40 gene expression independently of the proximal NF-κB signaling cascade. Because NF-κB recruitment to the IL-12 p40 gene promoter is increased in IL-10$^{-/-}$ BMDC (figure 3C), we hypothesized that STAT3 signaling interferes with recruitment of cRel to this gene promoter. To investigate this possibility, IL-10$^{-/-}$ BMDC cells were infected with Ad5STAT3C or Ad5GFP virus, stimulated with LPS for 4h and cRel recruitment to the IL-12 p40 gene promoter was analyzed by ChIP assays. As seen in figure 7B, Ad5STAT3C, but not Ad5GFP, blocked cRel recruitment to the IL-12 p40 gene promoter (figure 7B). Moreover, IL-10 pre-treatment inhibited LPS-induced cRel loading to the IL-12 p40 gene promoter (figure 7B). Altogether, these findings indicate that STAT3 controls LPS-induced IL-12 p40 gene expression by inhibiting recruitment of NF-κB subunits to the gene promoter, a process absent in IL-10$^{-/-}$ BMDC.

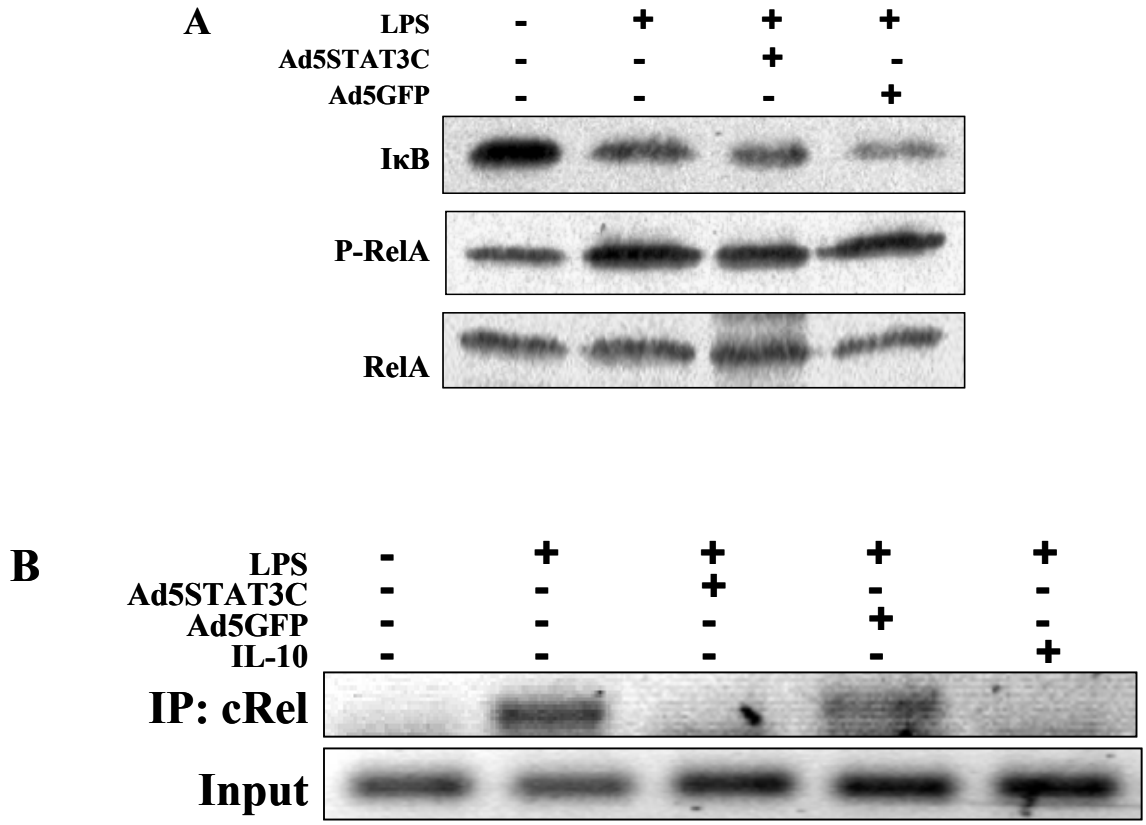

Figure 7. Adenoviral delivery of constitutively active STAT3 in IL-10$^{-/-}$ BMDC inhibits cRel recruitment to the IL-12 p40 gene promoter without affecting the NF-κB pathway. IL-10$^{-/-}$ BMDC were infected with either Ad5STAT3C or Ad5GFP, or preincubated with IL-10 (10 ng/ml) for 12 hours, and then stimulated with LPS (5 μg/ml) for 1 or 4h. (A) Protein extracts from infected BMDC were collected after one hour, and western blot analysis was performed using antibodies for IκB, phospho-RelA, and RelA. (B) Cells were treated for four hours as described above and cRel recruitment to the IL-12 p40 promoter was analyzed by ChIP, as described in the methods section. The results are representative of two independent experiments.

Discussion

Innate immune responses are induced by the presence of pathogenic and non-pathogenic microorganisms and lead to the activation of a complex gene program aimed at re-establishing host homeostasis. Although initiation of innate immunity is a critical feature of host homeostasis, failure to regulate and/or terminate this response can have deleterious consequences for the host. For example, IBD, which include Crohn's disease and ulcerative colitis, are associated with dysregulated innate and adaptive immune responses to luminal non-pathogenic bacteria.[37-40]

The immunosuppressive cytokine IL-10 exerts numerous immunoregulatory functions and plays a pivotal role in maintaining intestinal homeostasis and controlling innate responses.[39] This is shown in IL-10$^{-/-}$ mice that develop spontaneous intestinal inflammation when housed under specific pathogen-free conditions.[1,3] However, these mice remain healthy and disease free when born and raised under gnotobiotic conditions, suggesting that IL-10 is involved in regulating innate host responses to the luminal intestinal flora.[1] Despite numerous attempts, the molecular

mechanisms of IL-10 mediated regulation of innate immune responses have not been clearly elucidated.

In this study, we investigated the molecular mechanism of dysregulated innate responses in BMDC isolated from IL-10$^{-/-}$ mice. We report that LPS induced a stronger IL-12 p40 and TNFα gene expression in BMDC derived from IL-10$^{-/-}$ compared to wild-type mice. This indicates that in the absence of endogenous IL-10, LPS responsiveness is enhanced in BMDC, leading to higher IL-12 p40 and TNFα gene expression. Importantly, administration of neutralizing IL-12 antibody prevents the early onset of colitis in IL-10$^{-/-}$ mice.[5] Thus, our finding that LPS induced higher IL-12 p40 gene expression in BMDC isolated from IL-10$^{-/-}$ mice correlates with the key role for IL-12 p40 in this model of experimental colitis.

LPS-induced IL-12 p40 gene expression is regulated by various signaling cascades and transcription factors.[31,41,42] Among them, the NF-κB transcriptional system has been shown to play a preponderant role in IL-12 p40 gene expression. In an effort to understand the dysregulated IL-12 p40 gene expression in IL-10$^{-/-}$ BMDC, we carefully investigated LPS signal transduction in both wild-type and IL-10$^{-/-}$ mice. We found that LPS-induced IL-12 p40 gene expression is strongly inhibited in BMDC expressing an IκB super-repressor, showing the critical role for NF-κB in regulating this cytokine in BMDC. The negative effect of IL-10 on NF-κB signaling is controversial and may reflect cell type specificity. Interestingly, a new report showed that IL-10 blocked LPS-induced IKK activity and RelA phosphorylation in BMDC.[14] Surprisingly, using similar dendritic cells, we found that LPS-induced proximal NF-κB signaling is similar between wild-type and IL-10$^{-/-}$ BMDC, whereas IL-12 p40 gene expression is stronger in IL-10$^{-/-}$ cells. Indeed, levels of IκBα degradation/phosphorylation, RelA phosphorylation and NF-κB transcriptional activity were comparable between LPS-stimulated wild-type and IL-10$^{-/-}$ cells in our study. Thus, although NF-κB activity is essential for LPS-induced IL-12 p40 gene expression, this signaling cascade is not excessively activated in IL-10$^{-/-}$ BMDC. The discrepancy between results in these studies may be related to the supraphysiological dose of IL-10 used (50 ng/ml) by Bhattacharyya et al. For example, we found that low amounts of exogenous IL-10 in the physiologic range (1 ng/ml, compared to 0.7 ng/ml secreted by LPS-induced BMDC, figure 1B) totally blocked LPS-induced IL-12 p40 and TNF secretion without inhibiting NF-κB activity (not shown in present study). In addition, our study utilized cells lacking endogenous IL-10, which allowed physiological analysis of gene expression in the absence of the endogenous immunoregulatory IL-10 molecule. Also LPS-induced JNK and p38 phosphorylation is similar in wild-type and IL-10$^{-/-}$ BMDC, suggesting that LPS induced TLR-4 signal transduction is not impaired in either wild-type or IL-10$^{-/-}$ BMDC.

IL-10 has been shown to directly inhibit NF-κB activity through transient blockade of IκB degradation and IKK activity as well as impaired NF-κB DNA binding activity in LPS-stimulated monocyte cell lines.[13] Interestingly, we found no evidence of IL-10-mediated inhibition of IκB degradation and phosphorylation, suggesting that this immunosuppressive cytokine acts through a different mechanism in murine BMDC. This is in agreement with previous studies showing no effect of IL-10 on NF-κB activation[43] and MAP kinases[44] in human macrophages. This data suggests that the positive signaling cascade leading to increased IL-12 p40 gene expression is not dysregulated in IL-10$^{-/-}$ BMDC. Thus, impaired activation of inhibitory signaling cascades in IL-10$^{-/-}$ cells may lead to dysregulated innate host responses in these mice.

Persistent and sustained recruitment of transcription factors to selective gene promoters is responsible for prolonged gene expression. Indeed, kinetics of NF-κB dependent gene transcription directly correlates with the extent and duration of recruitment of various subunits to gene promoters.[34] Of considerable interest, we showed for the first time enhanced recruitment of

cRel to the IL-12 p40 promoter in LPS-stimulated IL-10$^{-/-}$ BMDC but not wild-type cells. Moreover, cRel was still associated with the IL-12 p40 promoter at eight hours following LPS stimulation in IL-10$^{-/-}$ BMDC, whereas no such binding was observed in wild-type BMDC. Thus, although proximal NF-κB signaling is similar between IL-10$^{-/-}$ and wild-type BMDC, recruitment of NF-κB to the IL-12 p40 gene promoter is clearly different. Therefore, both enhanced initial binding of cRel and failure to remove NF-κB from the IL-12 p40 promoter rather than excessive proximal signaling is responsible for enhanced gene expression.

Negative regulators of LPS signaling play a pivotal role in controlling innate responses in numerous immune cells.[39] Interestingly, we found that STAT3, a negative regulator of LPS signaling, is strongly phosphorylated in endotoxin-stimulated wild-type but not in IL-10$^{-/-}$ BMDC. This prolonged and strong STAT3 phosphorylation in wild-type BMDC is likely due to increased IL-10 production. First, LPS induced both IL-10 gene expression and strong STAT3 phosphorylation in wild-type but not in IL-10$^{-/-}$ BMDC. Second, IL-10 receptor blocking antibody prevented LPS-induced STAT3 phosphorylation in wild-type BMDC. Thus, STAT3 phosphorylation is mediated by LPS-induced IL-10 in wild-type BMDC. The lack of STAT3 phosphorylation in IL-10$^{-/-}$ cells is functionally linked to enhanced IL-12 p40 gene expression. This is clearly illustrated in the experiment where we delivered a constitutively active STAT3 in IL-10$^{-/-}$ BMDC using an adenoviral vector. Using this approach, we demonstrated that LPS-induced IL-12 p40, but not TNFα gene expression is strongly inhibited in IL-10$^{-/-}$ BMDC. Conversely, blocking JAK/STAT signaling in wild-type BMDC enhanced LPS-induced IL-12 p40 protein secretion. Thus, STAT3 has a critical role in down regulating LPS-induced IL-12 p40 gene expression. This is a selective inhibition, so this mechanism is not responsible for suppressing other proinflammatory cytokines induced by LPS, such as TNFα.

In this study, we provide clear evidence that the STAT3 pathway controlled cRel recruitment to the IL-12 p40 gene promoter, thereby providing a mean to selectively terminate gene transcription. First, we showed that Ad5STAT3C prevents cRel loading to the IL-12 p40 gene promoter without interfering with LPS-induced proximal NF-κB signaling. Second, IL-10 mediated blockade of LPS-induced IL-12 p40 gene expression occurs independently of proximal NF-κB signaling but rather involves decreased cRel recruitment to the gene promoter. How can STAT3 activation prevent NF-κB recruitment to the IL-12 p40 gene promoter independently of proximal signal transduction? One possible scenario is that activated STAT3 migrates to the nucleus and interferes with a molecule involved in the regulation of IL-12 p40 gene expression. This would be consistent with the partial nuclear localization of activated STAT3 in Ad5STAT3C-infected IL-10$^{-/-}$ BMDC. Interestingly, STAT3 directly interacted with RelA and suppressed IL-1β and LPS/interferon-γ induced iNOS gene expression in mesangial cells.[45] However, using co-immunoprecipitation analysis, we were unable to detect STAT3/NF-κB interaction in wild-type or IL-10$^{-/-}$ BMDC. Another potential mechanism could involve STAT3 signaling interference with chromatin remodeling through alteration of histone acetylation and/or phosphorylation. Nucleosome remodeling of nucleosome 1, which contains the Rel and C/EBP cis-elements, is a critical event for signal-induced IL-12 p40 gene expression.[46] We have previously reported that the TGF-β/Smad pathway blocked LPS-induced histone phosphorylation/acetylation in intestinal epithelial cells.[26] It is interesting to speculate that IL-10 activates an event through STAT3 that leads to impaired chromatin remodeling and decreased recruitment of essential transcription factors such as cRel and C/EPB. However, Zhou et al demonstrated that IL-10 induced only slight changes in chromatin remodeling and C/EPB recruitment to the IL-12 p40 promoter in LPS-stimulated peritoneal macrophages.[47] Moreover, IL-10 failed to block LPS-induced histone-3 phosphorylation (ser 10) in the present study (data not

shown). Whether other histone modifications are affected by IL-10 is currently under investigation. The impact of STAT3 signaling events on chromatin remodeling in BMDC remains to be determined. Nevertheless, activation of the IL-10/STAT3 pathway likely controls the initial recruitment of transcription factors (cRel, RelA and probably others) and/or stability of protein/DNA interaction, which ultimately dictate the amount of IL-12 being transcribed by BMDC.

It is interesting to note that STAT3 blocks LPS-induced IL-12 p40 but not TNFα gene expression. Such specific effect has been previously reported for Bcl-3 that is required for inhibition of LPS-induced TNFα but not IL-6 secretion in macrophages.[18] Moreover, IL-10-induced NF-κB p50 DNA binding activity is responsible for blockade of TNFα-induced IL-6 and MIP2α gene expression in peritoneal macrophages.[48] Thus, the molecular mechanism of IL-10-mediated gene inhibition is diverse and cell type specific. This also indicates that STAT3 selectively altered a critical component of the transcriptional machinery involved in IL-12 p40 expression.

The recently discovered cytokine IL-23 consists of the IL-12 p40 and p19 subunits and is secreted by immune cells including dendritic cells.[6] This cytokine has the ability to skew T cell differentiation towards a T_H1 profile. Interestingly, IL-23p19 mRNA levels are increased in experimental colitis[49] and in Crohn's disease patients.[50] Moreover, mice over expressing IL-23p19 develop multiple organ failure, systemic inflammation and die before three month of age.[51] Since LPS-induced IL-12 p40 gene expression is reduced by Ad5STAT3C, the decreased production of this subunit may not only impair IL-12 secretion but may also reduce the production of IL-23 in BMDC. Further studies will be necessary to understand the impact of STAT3 and IL-23 expression on inflammatory diseases.

Our study highlights the complex network of regulatory cascades involved in controlling LPS-induced signaling in immune cells. Clearly, malfunction in these regulatory pathways impairs host homeostasis. First, tissue-specific disruption of STAT3 in bone marrow cells during hematopoeiesis led to overly activated innate immune responses and Crohn's disease like intestinal inflammation.[21] The *in vivo* relevance is shown by the severe intestinal inflammation caused by deletion of STAT3[20,21] or IL-10.[2] Conversely, intestinal bacteria[52] and regulatory T cells,[53] engineered to produce IL-10, prevent and treat inflammation in murine models of experimental colitis.

In conclusion, we show that increased LPS-induced IL-12 p40 gene expression in IL-$10^{-/-}$ BMDC involves dysregulated recruitment of cRel to the IL-12 p40 promoter due to defective STAT3 activation. We propose a model where LPS-induced IL-10 production leads to the activation of STAT3 and inhibition of IL-12 p40 gene expression by preventing NF-κB access to the gene promoter. This negative feedback mechanism is essential for the attenuation of LPS-induced innate responses in immune cells. These data provide a potential molecular mechanism for inflammation in IL-$10^{-/-}$ mice. Therefore, enhancing STAT3 signaling may represent a potential therapeutic means to manipulating the innate immune system and treating intestinal inflammation.

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10 General Discussion

Introduction

Genetic susceptibility and environmental factors are required for a host to develop IBD, as outlined in the general introduction. Each separate component alone is not sufficient to induce disease, whereas the combination of genetic susceptibility and intestinal commensal bacteria synergistically determine onset and perpetuation of colitis.

The animals used in the experiments as described in this thesis, HLA-B27 transgenic rats and IL-10$^{-/-}$ mice, allow the study of the immune responses to bacteria in genetically susceptible animals. The results from these studies are briefly discussed in the following paragraphs. Animal models mimic, but not always accurately reflect the characteristics of human IBD. However, many findings from animal models can be translated to IBD patients. Therefore, the discrepancies and relevance of our findings for the pathogenesis and treatment of human IBD are emphasized in this chapter. Furthermore, some future directions are included, as discussed per chapter.

Attenuation of colitis by manipulating the intestinal microflora

Antibiotics

Compelling data exists that bacteria and their products play a crucial role in the pathogenesis of chronic intestinal inflammation in animal models of experimental colitis and human IBD.[1,2] Crohn's disease is most commonly found in regions of the gastrointestinal tract that harbor high concentrations of enteric microflora, such as the terminal ileum and colon. Decreasing the exposure to bacteria by broad spectrum antibiotic therapy, or fecal deviation, attenuates colitis. Recurrence of disease can be induced by re-introduction of intestinal contents into the bypassed distal intestinal tract. Thus, based on the important role for bacteria in the pathogenesis of intestinal inflammation, there is a rationale for the use of antibiotics as primary or maintenance therapy in IBD.[3]

In Crohn's disease, metronidazole[4,5] and metronidazole in combination with ciprofloxacin[6-8] are effective in treating colonic inflammation. Also fistulizing Crohn's disease and pouchitis respond to metronidazole.[9-12] Although less convincing, there is a role for ciprofloxacin in treating Crohn's disease.[13,14] The use of antibiotics in ulcerative colitis is currently not justified, based on available studies.

In this thesis, we concluded in chapter 3 that ciprofloxacin is most effective in treatment of cecal inflammation; metronidazole preferentially treats the colon, whereas vancomycin-imipenem definitively treats both regions. Importantly, these results suggest that subsets of aerobic or anaerobic bacteria show regional differences in their capacity to mediate experimental colitis in IL-10$^{-/-}$ mice.

Various studies in animal models indicate that commensal bacterial strains selectively induce inflammation in specific parts of the intestinal tract. For example, IL-10$^{-/-}$ mice monoassociated with *E. coli* preferentially develop cecal inflammation with an early onset of disease.[15] In contrast, monoassociation of IL-10$^{-/-}$ mice with *E. faecalis* induces distal colitis with slow onset of disease, starting at 12 weeks of colonization.[15,16] These data support the important role of intestinal bacteria for the pathogenesis of IBD and provide therapeutic potential for manipulating the intestinal flora to influence the onset and course of disease. The implications for

IBD patients could be that the composition of the microbiota at the various locations in the intestinal tract can determine the phenotype of disease. A better understanding of the intestinal bacterial profile for individual patients and their regional distribution is required to fully investigate this hypothesis. Subsequently, selective antibiotic therapy could reduce disease-inducing bacteria in affected regions, depending on the phenotype of disease.

Interestingly, we detected greater efficacy of selective antibiotics for preventing rather than treating colitis. This may be explained by different roles for various endogenous bacterial species in the different phases of the inflammatory process. Some bacterial species might initiate inflammation while a larger spectrum of intestinal bacteria perpetuates disease.[17] Therefore, it seems easier to prevent onset of colonic inflammation than to treat established disease. In human IBD, based on the latter hypothesis, antibiotics would be more beneficial as maintenance therapy after remission has been established. Disadvantages of the long-term use of antibiotics include bacterial resistance to antibiotics as well as bacterial overgrowth. Therefore, combination therapy with other therapies that change the bacterial microflora, such as probiotic and prebiotic therapy, will be required to prevent these unwanted effects.

In conclusion, based on our and other studies, there is a potential role for antibiotics in the treating Crohn's disease. Our data suggest that broad spectrum agents will be more successful than narrow spectrum antibiotics. The type and duration of antibiotics can be dependent on the location of disease, species of disease-inducing bacteria, and the genetic background of the host. The combination with probiotic and prebiotic therapy, with or without antibiotic pretreatment, is an attractive option that needs further investigation.

Probiotics

Probiotic organisms can potentially interact with or affect three distinct yet interrelated components within the intestinal milieu: epithelial cells, enteric flora, and/or mucosal immune cells. Conventional therapy for IBD usually targets only one component of disease pathogenesis, the mucosal inflammatory response.[18] Since the majority of standard treatment regimens are either ineffective in completely eradicating disease or are hindered by significant side effects, there is a need to identify and characterize novel pharmacologic therapies that will effectively treat and maintain remission of these inflammatory disorders.

Probiotics are living commensal microorganisms that are important to the health and well being of the host.[19] The most convincing evidence for the beneficial effect of probiotics in IBD so far is derived from a clinical trial with VSL#3. VSL#3 contains $9x10^{11}$ viable probiotic organisms, including four strains of *Lactobacilli*, three strains of *Bifidobacteria*, and one strain of *Streptococcus salivarius*. This probiotic cocktail was very effective in preventing pouchitis after antibiotics-induced remission.[20] Preliminary uncontrolled studies suggest that this agent may be effective in treating ulcerative colitis patients.[21,22] Single probiotic species have also been identified and shown to improve disease in ulcerative colitis patients, as shown for the *E. coli* 1917 Nissle.[23,24] However, *Lactobacillus GG* failed to induce remission in Crohn's disease patients in a small pilot-study.[25]

In chapter 4, a clinically relevant finding of our study was that oral *Lactobacillus GG* treatment partially prevented relapse of colitis in HLA-B27 transgenic rats after antibiotic treatment, whereas *Lactobacillus GG* administration alone failed to show a beneficial effect. This conclusion was supported by significantly decreased cecal inflammation and colonic histology scores, as well as a significant decrease of inflammatory markers and cytokines in antibiotic-*Lactobacillus GG*-treated transgenic rats versus other treatment groups. This is the first report of a synergistic effect of antibiotic and probiotic therapy in experimental colitis.

A possible explanation for the synergistic effects of the combination of antibiotic and probiotic treatment is that the combination treatment promoted synergistic interaction of *Lactobacillus GG* with other endogenous probiotic species, resulting in disease protection. The absence of these potential beneficial interactions with other intestinal protective bacteria in gnotobiotic transgenic rats co-associated with *Lactobacillus GG* and *B. vulgatus* could also explain the lack of efficacy of *Lactobacillus GG* in prevention of colitis. Another possibility of the beneficial effects of the combination treatment in HLA-B27 transgenic rats is the transient decrease of competing bacteria by antibiotics, opening an environmental niche for probiotic bacteria. Translated to human IBD, restoring the microbial balance between detrimental and protective luminal bacteria by combining antibiotic and probiotic approaches may be the most physiologic approach to treat IBD and may alter the natural history of these chronic relapsing diseases.

Another major finding in the antibiotic-*Lactobacillus GG* study was the prevention of colitis relapse by specific *Lactobacillus* species as demonstrated by the lack of efficacy of *Lactobacillus plantarum* in preventing colitis relapse in transgenic rats after antibiotic treatment. However, this result is in contrast with our previous reports that oral *Lactobacillus plantarum* attenuated colitis in IL-10$^{-/-}$ mice,[26] whereas *Lactobacillus GG* had no effect.[27] These contrasting responses suggest that different *Lactobacilli* may have variable host specificity or different efficacy in various inflammatory conditions, indicating that not all probiotics are equally protective in chronic experimental colitis, and perhaps IBD patients. The concept of selective dependency of beneficial effects of a probiotic bacterial species on the species and genetic background of the host is similar to that of disease-inducing bacteria.[16,28-30] Extrapolated to human IBD this suggests that not all patients will respond equally to probiotic therapy and that each patient may respond to a different probiotic preparation. Genetic background, location of disease, and the pre-existent microflora profile, could determine optimal protective agents for individual patients. This study already identified a group of patients that can benefit from probiotic therapy: antibiotic-treated patients. Further identification of subgroups of patients, selected by phenotype of disease or genetic background, and possibly intestinal microflora profiles, might be necessary to fully utilize the beneficial effects of probiotics in the treatment of IBD.

Recent studies have shown that the beneficial effects of the probiotic combination VSL#3 are mediated by TLR-9.[31,32] These studies showed the attenuation of experimental colitis by oral administration of VSL#3-extracted DNA.[31,32] A conclusion from the latter study was that "live microorganisms are not required to attenuate experimental colitis." This conclusion was drawn because the administration of DNA or non-viable bacteria also achieved amelioration of colitis in murine models of experimental colitis. The implication could be that VSL#3 is not a probiotic, since the definition of a probiotic is "the living commensal microorganisms that are important to the health and well being of the host."[19] However, the exciting concept of treatment of colitis using bacterial DNA rather than live bacteria is based on studies in animal models and needs further investigation and confirmation in clinical trials.

In conclusion, we have shown that probiotic therapy prevents colitis after antibiotic treatment. Efficacy of treatment is dependent on the type of probiotic, the genetic background of the host, phenotype of disease, presence of endogenous microflora and synergy with additional therapies such as antibiotics. In the future, optimal conditions for probiotics as effective therapy need to be elucidated. For use in human IBD, details of interest should include the choice of probiotic species, synergistic effects with other therapies such as antibiotics, and specification of phenotypic and genetic subsets of patients.

Prebiotics

In chapter 5, prebiotics partially prevented colitis in HLA-B27 transgenic rats, which correlated with increased endogenous intestinal *Bifidobacteria* and *Lactobacilli* as shown by PCR-DGGE and FISH. Prebiotic treatment also induced immunomodulatory effects, with decreased mucosal proinflammatory IL-1β and increased immunomodulatory molecules such as TGF-β. These results provide insight into the protective mechanisms utilized by prebiotics to mediate their beneficial effects, and show promise for prebiotics as adjuvant maintenance therapy for chronic inflammatory bowel diseases. This approach could be a safe, easy to administer, and cost-effective alternative for current treatments in IBD. However, the current clinical support for prebiotic use in IBD patients is poor.

Only few small clinical trials have demonstrated the efficacy for prebiotics in human IBD. For example, germinated barley food extracts decreased clinical and endoscopic inflammation in mild-moderate ulcerative colitis.[33,34] Furthermore, inulin administration to ulcerative colitis patients with pouchitis after colectomy led to a reduction of inflammation in the pouch mucosa.[35] However, large multicenter clinical trials are required to convincingly support a role for prebiotic therapy in IBD.

This is the first study in which prebiotics were used as primary therapy in genetically engineered rodents. The results are in agreement with findings by Schultz et al in a recent study, who reported that a probiotic preparation that also contained inulin attenuated colitis development in HLA-B27 rats.[36] In another study, the application of the probiotic combination of *Lactobacillus GG* and *Bifidobacterium lactis*, and the prebiotic combination of inulin and oligofructose, or the prebiotic- plus probiotic combination was tested in rats.[37] The prebiotic combination increased IL-10 secretion in Peyer's patches, whereas sIgA secretion was increased by both prebiotic and the combination treatment. No significant effect on NK cells activity or spleen and mesenteric lymph node cells were detected. Although the different treatments affected immunomodulation, no additive or synergistic effects were observed.[37] Thus, although there is rationale for the use of prebiotics in synergy with probiotics (synbiotics), this concept is not (yet) supported by studies in models of experimental colitis.

The negative side-effects of prebiotics are relatively unknown. However, it is well documented that prebiotic therapy profoundly changes the intestinal microflora, as shown in chapter 5. In addition, not only luminal bacteria, but also the mucosa-associated gut flora is affected by the prebiotic combination oligofructose/inulin. This was shown by increased mucosal concentrations of *Lactobacilli* and *Bifidobacteria* in the proximal and distal colon, when endoscopically obtained samples were cultured.[38] Of interest, mucosa-associated concentrations of cultured anaerobes and *Bacteroides* spp. were not affected by the prebiotic treatment. The potential for prebiotics to stimulate growth of not only beneficial but also pathogenic bacterial strains was demonstrated by a study of *Salmonella* species. Both inulin and fructo-oligosaccharide impaired resistance to intestinal *Salmonella enterica* in rats, which was corrected after calcium supplementation.[39] This would imply that prebiotics can also increase the risk of pathogenic bacterial growth in susceptible hosts. Another unwanted effect of prebiotic therapy such as lactulose is diarrhea, a common side-effect of this drug.

As a whole, evidence for the beneficial effects of prebiotic therapy is emerging in animal models and clinical trials. The potential advantages of prebiotic therapy (few side-effects, cost-effective, natural components) justify future mechanistic research and large well-designed clinical trials to support the potential use of prebiotics as adjuvant maintenance therapy.

To summarize part I, therapeutic manipulation of the intestinal microflora has clear advantages over conventional medications. Changing intestinal microbiota focuses on initial

disease pathogenesis, has fewer side effects, and has the potential to change the natural course of disease versus potentially toxic standard therapy. The genetic background of the host can determine the choice of therapy, since different bacterial strains have selective activities dependent on the genetic background of the host.[2] As pointed out in the introduction, future identification of susceptibility genes for IBD will allow us to divide patients into different subgroups with optimal treatment strategies for each group. For example, if genetic testing indicates an expected colonic phenotype of disease, metronidazole might be used for prophylaxis, followed by probiotic treatment. It is possible that different bacterial subsets induce disease in various intestinal regions in IBD patients, as suggested by monoassociated IL-10$^{-/-}$ mice.[15] Therefore, antibiotics, probiotics, prebiotics, and/or the combination of two or all three of these agents, have great potential of becoming well-accepted therapeutic strategies in the treatment of IBD. However, successful use of this approach will require thoughtful identification of patient subgroups that will selectively respond to a therapeutic cocktail.

Bacterial-induced immune responses in experimental colitis

Adaptive immune responses

T lymphocytes

In Part II, we studied innate and adaptive immune responses to bacteria and their products in HLA-B27 transgenic rats and IL-10$^{-/-}$ mice. Using these models of experimental colitis, we first explored the cytokine profiles in mesenteric lymph node cells after stimulation with a lysate from cecal contents. Transgenic HLA-B27 transgenic rats showed an increased ratio of pro- versus anti-inflammatory cytokine secretion.

In chapter 6 we showed that T cells are critical for the production of the proinflammatory cytokines IFN-γ and IL-12 in HLA-B27 transgenic rats. In several other genetically engineered animal models T cells are crucial to the development of inflammation. This has been demonstrated in IL-2$^{-/-}$ mice,[40] IL-10$^{-/-}$ mice,[41] TCRα$^{-/-}$ mice,[42] Tgε$_{26}$ mice,[43] and CD45RB^{high} T cells transfer into either SCID mice[44] or athymic rats.[45] However, all of these studies were performed in specific pathogen-free conditions. To study the role of T cells in a disease-susceptible host monoassociated with a single commensal bacterial strain, we monoassociated *rnu/rnu* or *rnu/+* transgenic rats with *B. vulgatus*. *B. vulgatus* is a non-pathogenic bacterial strain that induces mild colitis in HLA-B27 transgenic rats.[28] In chapter 7, we used nude athymic HLA-B27 transgenic rats. Colitis developed in *rnu/+* transgenic rats monoassociated with *B. vulgatus*, but *rnu/rnu* transgenic rats remained disease free in the absence of T cells. In the latter group of rats, transfer of CD4$^+$ T cells induced disease, confirming that CD4$^+$ T cells are required for induction of colitis.[46] Thus, T cells are critical for the development of intestinal inflammation, not only in specific pathogen-free conditions, but also in monoassociation with *B. vulgatus*.

The question remains whether T cell responses to *B. vulgatus* in the latter model are antigen-specific. This could be addressed in future experiments in rats monoassociated with *E. coli*, which remain disease free. Transfer of CD4$^+$ T cells from *B. vulgatus* monoassociated *rnu/+* transgenic rats into *E. coli* monoassociated *rnu/rnu* transgenic rats could address this hypothesis. If colitis develops in *E. coli* monoassociated *rnu/rnu* transgenic recipients, we can reject the hypothesis of antigen-specificity.

Bacterial specific T cell responses found in animal models are relevant for human IBD, considering that T-cell clones from human mucosal biopsies showed specific responses to selective commensal bacteria, including *Bacteroides*, *Bifidobacteria*, and *E. coli*.[47,48] Several studies have attempted to elucidate the mechanisms of bacterial-specific T cell clones in animal models. For example, adoptive transfer of bacterial-antigen-activated $CD4^+$ T cells from colitic C3H/HeJBir but not from control C3H/HeJ mice to C3H/HeSnJ SCID recipients induced colitis.[49] In another study, a model of antigen-specific colitis was developed by transfer of OVA-specific T cells from RAG-$2^{-/-}$ TCR transgenic mice into RAG-$2^{-/-}$ recipients whose intestinal tracts were colonized with OVA-expressing or control *E. coli*.[50] In this model, transfer of polarized T_H1 and T_H2 subsets induced colitis in recipients colonized with OVA-expressing but not control *E. coli*, and induction of disease was dependent on bacterial-associated OVA. These results indicate the role of a specific bacterial antigen in the onset of colitis.[50] In a recent study, flagellins, which are molecules known to activate innate immunity via TLR-5, were identified as dominant antigens in experimental colitis by screening an expression library of enteric bacteria for serologic responses.[51] In this study, multiple strains of colitic mice had elevated serum anti-flagellin IgG2a responses and T_H1 T cell responses to flagellin. In addition, flagellin-specific $CD4^+$ T cells induced severe colitis when adoptively transferred into naive SCID mice. Serum IgG to these flagellins was elevated in Crohn's disease patients, but not in patients with ulcerative colitis or in controls.[51] The latter study identified flagellins as a bacterial product that functions not only as an adjuvant but also as an antigen that stimulates pathogenic intestinal immune reactions in genetically susceptible hosts. Taken together, the data discussed in this paragraph indicate that T cells reactive with the enteric bacterial flora and their products can mediate chronic intestinal inflammation.

However, immunoregulatory responses can also be antigen-specific. For example, bacterial-reactive T regulatory cells prevented onset of colitis in C3H/HeSnJ SCID recipients in co-transfer with T_H1 T cells reactive to intestinal commensal bacteria.[52] In another study, *H. hepaticus* induced colitis in IL-$10^{-/-}$ but not in wild-type mice.[53] Transfer of $CD4^+$ T cells from IL-$10^{-/-}$ mice to RAG$^{-/-}$ mice induced colitis, which could be prevented by co-transfer of $CD4^+$ T cells from *H. hepaticus*-infected but not uninfected wild-type mice. This protective effect was mediated by IL-10 secretion by $CD4^+$ T cells in a *H. hepaticus*-antigen-specific manner.[53]. However, regulatory T cells can also exert their protective effects in a non-antigen-specific fashion. $CD4^+$ T cells engineered to secrete IL-10 can home to the gut, and prevented colitis induced by transfer of $CD45RB^{hi}$ cells into SCID mice.[54,55] Thus, bacterial- and antigen specificity is an important feature of the immune system, and antigen-specific T cells have distinct roles in pathogenic or regulatory immune responses. Characterization of specific subsets of T cells reactive to specific commensal bacteria could be a first step in defining targets for treatment of human IBD.

B lymphocytes

Generally, regulatory T cells are thought to secrete the majority of IL-10.[56,57] Of interest, this thesis shows that B cells were the major producers of the regulatory cytokines IL-10 and TGF-β in HLA-B27 transgenic rats. However, our finding that B cells produce regulatory cytokines confirmed findings by Mizoguchi et al, who demonstrated that IL-10-producing B cells are protective, since B cell/TCRα double deficient mice had more colitis than TCRα deficient mice with competent B cells.[58,59] The immunoregulatory role of IL-10 producing B cells was further demonstrated by the observation that transfer of B cells from IL-10/TCRα double deficient mice was unable to suppress chronic intestinal inflammation in B cell/TCRα double deficient mice.[58] The anti-inflammatory B cell subset in this model expressed high levels of CD1d, a

marker of B-1 B cells. Also in mouse spleens, B cells were detected that produce IL-10. These $B220^+CD19^+$ cells were shown to stimulate naive T cells, and might be involved in activating regulatory immune responses.[60] A gene that has been shown to be required for the development of IL-10-producing B cells is Gαi2.[61] Reconstitution of $RAG2^{-/-}$ mice with $G\alpha i2^{-/-}$ bone marrow induced an IBD-like colitis, accompanied by a selective deficit in IL-10 secreting B-1 B cells and an increase in B-2 B cells.

In contrast, B cells also exacerbate inflammation in some models. The transfer of $CD4^+$ T cells into SCID mice induces ileitis, whereas co-transfer of mesenteric lymph node B cells from SAMP1/YitFc mice increases the severity of ileitis compared to the transfer of $CD4^+$ T cells alone.[62] The suggested mechanism is the interference of SAMP1/YitFc B cells with the suppression of effector T cell proliferation by regulatory $\alpha_E\beta_7^+CD4^+$ T cells, thus contributing to the development of ileitis in SAMP1/YitFc mice. In another study, B cells were shown to trigger intestinal inflammation using transgenic mice expressing CD40 ligand ectopically on B cells (CD40L/B).[63] CD40L/B transgenic mice developed spontaneous severe transmural intestinal inflammation in both colon and ileum, whereas CD40L/B CD40 double-mutant mice did not develop colitis, indicating the direct involvement of CD40-CD40L interaction. Furthermore, mice transferred with $B220^+$ B cells from diseased CD40L/B transgenic mice and $CD4^+$ T cells from wild-type mice also develop colitis.

Controversy still exists on the role of B cells in the pathogenesis of experimental colitis and human IBD. IBD patients have increased serum and mucosal antibody responses to several commensal bacteria, including *E. coli*.[64-66] Furthermore, B-1 cells produce IgM or IgA in a T cell–independent manner, with specificities for common enteric bacterial antigens. However, other reports suggested that B cells only play a minor role in the pathogenesis of IBD. One study that supported the latter hypothesis showed that the severity of colitis that develops in $IL\text{-}10^{-/-}$ mice is similar to the colitis that developed in $B^{-/-}IL\text{-}10^{-/-}$ mice, implying that B cells were not the primary mediator of experimental colitis in this model.[41] As described previously in this chapter, the distinct effects of B cells per subset are important. Mucosal IFN-γ secreting B-2 cells induce proinflammatory effects and IL-10 producing B-1 cells induce anti-inflammatory responses.[67] Furthermore, the location of disease is relevant, since $CD45RB^{hi}$ T cells from $IFN^{-/-}$ mice can induce ileitis when transferred into SCID mice only when co-transferred with B cells,[68] suggesting that the proinflammatory activity of intestinal B cells may be particularly relevant in the ileum. Thus, specific subsets of B cells can have either pro or anti-inflammatory capacities, depending on their phenotype. These results are relevant to the pathogenesis of IBD and imply that reducing (B-2) or stimulating (B-1) B cells could be a potential strategy for treating IBD.

Antigen-presenting cells

After establishing the role of T cells in *B. vulgatus* induced colitis in *rnu/rnu* HLA-B27 transgenic rats, we investigated the role of antigen-presenting cells in controlling T cell function. As described in chapter 8, HLA-B27 transgenic *rnu/rnu* recipients receiving either non-transgenic or transgenic cells developed more severe intestinal inflammation compared to *rnu/rnu* non-transgenic recipients receiving either transgenic or non-transgenic cells. Therefore, we concluded that T cells from either non-transgenic or transgenic donors induce colitis in *rnu/rnu* transgenic but not in non-transgenic recipient rats, suggesting that activation of T cells by antigen presenting cells expressing HLA-B27 is pivotal to the pathogenesis of colitis in this model.

The role of antigen-presenting cells in determining the nature of the immune response has been extensively evaluated, and the results indicate that antigen-presenting cells are crucial for both activation and also for subsequent down-regulation of immune responses.[69] The results of

our *in vivo* transfer study indicate that accessory cells in *rnu/rnu* transgenic recipients may lack the capacity to inhibit the colitis-inducing abilities of $CD4^+$ T cells from donor rats that interact with HLA-B27 expressing cells. Defective stimulation of T cells by antigen-presenting cells in HLA-B27 transgenic rats was recently confirmed.[70] One of the underlying mechanisms described in this study involves a decreased use of the costimulatory molecule CD86 by HLA-B27 dendritic cells. Impaired costimulatory function could result in a loss of tolerance toward microbial flora in this model. However, activation of T cell responses also plays a role in the HLA-B27 transgenic rat model. The importance of HLA-B27 in the activation of T cell responses was underlined by our observation that addition of anti-HLA-B27 antibodies to co-cultured transgenic $CD4^+$ T cells plus cecal bacterial lysate-pulsed transgenic antigen presenting cells reduced IFN-γ responses.[71] Thus, the activation of antigen-presenting cells by commensal bacterial products can induce both overly aggressive immune responses and can also lead to a loss of tolerance in the genetically susceptible host.

The findings of the study presented in chapter 8 emphasize the importance of antigen-presenting cell function in regulating host immune responses to commensal bacteria in experimental colitis, and also indicates that the antigen-presenting cell might be of value as a therapeutic target to treat IBD and spondyloarthropathies. Some therapeutic approaches for human IBD are directed against T cell function. Although the T cells are important tools for the induction and perpetuation of colitis, antigen-presenting cells represent a diverse group of cells that can determine the outcome of bacterial-induced T cell responses.

Innate immune responses

Dendritic cells form a small subset of cells that have potent immunoregulatory capacities. Mucosal dendritic cells actively process bacterial products and regulate immune responses by transporting these antigens to mesenteric lymph nodes and thereby inducing T cell responses. Secondly, dendritic cells are present at the interface of innate and adaptive immunity by virtue of their ability to secrete various cytokines including TNFα, IL-12 p40 and IL-23.[72,73] For instance, IL-12 p40 producing dendritic cells skewed T cell differentiation towards a T_H1 profile, a hallmark in the IL-$10^{-/-}$ experimental mouse model.[74,75] Immunoregulatory cytokines, such as IL-10, can subsequently influence if dendritic cells induce either pro- or anti-inflammatory responses.

Reported responses to systemic administration of IL-10 have been poor in human Crohn's disease.[76,77] One possible explanation for this lack of effect is the absence of high concentrations of IL-10 at the relevant sites, such as the intestinal mucosa and mesenteric lymph nodes. Indeed, when high amounts of IL-10 were delivered at the site of inflammation, experimental colitis could be attenuated. This was demonstrated using IL-10 producing *Lactococcus lactis*[78] and regulatory T cells,[54] engineered to produce IL-10 and to home to the site of inflammation.[55,78] Both studies showed that the local delivery of IL-10 prevented and treated intestinal inflammation in murine models of experimental colitis. Alternatively, understanding of the mechanisms of IL-10 mediated effects are required to determine alternative strategies for recombinant IL-10 treatment.

In chapter 9, we report mechanisms of IL-10-mediated innate immune responses and showed that IL-10-induced STAT3 interfered with the recruitment of transcription factors to the gene promoter of the proinflammatory cytokine IL-12 p40. Previously, the importance for IL-10-induced STAT3 signaling for host homeostasis was shown by deletion of STAT3, that was embryologically lethal.[79] Moreover, myeloid cell-specific STAT3-deficient mice developed severe enterocolitis,[80] and STAT3 deletion in bone marrow-derived cells lead to overly activated innate immune responses[81] and interfered with the adaptive immune system by inhibiting the induction of antigen-specific T cell tolerance.[82]

In animal models of experimental colitis, but also in IBD patients, the presence of intestinal bacteria and their products leads to a continuously activated immune system. Subsequent NF-κB-mediated proinflammatory immune responses can determine the onset and perpetuation of colitis in the genetic susceptible host. Mechanisms of interference with NF-κB activation are therefore crucial for the development of therapeutic strategies in IBD. Based on our findings, we propose a model in which LPS-induced IL-10 production leads to the activation of STAT3 and inhibition of IL-12 p40 gene expression by preventing NF-κB access to the gene promoter. This negative feedback mechanism is essential for the attenuation of TLR-4 mediated LPS-induced innate responses in immune cells. Importantly, Haga et al showed protection of Fas-mediated liver injury by overexpression of constitutively active STAT3 following intravenous administration.[83] Thus, enhancing STAT3 signaling may represent a potential therapeutic target to manipulate the innate immune system and to treating IBD.

Conclusions

Enormous progress has been made in the past decade to elucidate the mechanisms of chronic intestinal inflammation as seen in IBD. Novel techniques provided the tools to identify genetic mutations, determine the role of specific subsets of immune cells in inflammation, and map the intestinal microflora and their responses to therapy. The various ongoing clinical trials translate current basic laboratory findings to potential new therapeutic strategies aimed at improving the natural course of disease in IBD patients. Basic research is and will remain the cornerstone of clinical progress in inflammatory bowel diseases and will lead to novel future therapies that selectively target defined patient subgroups. A better understanding of the mechanisms by which commensal bacterial adjuvants and antigens stimulate pathogenic and protective immune responses will help identify optimal subgroups for treatment, therapeutic approaches, and combinations of therapies.

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Summary
Samenvatting

Summary

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, have a great impact on their victims' quality of life. Our knowledge about these diseases is limited and thus treatment is as well. In an effort to better understand the causes of IBD, this thesis addresses (1) the attenuation of bacterial-induced experimental colitis by manipulating the intestinal microflora, and (2) the regulation of bacterial-induced innate and adaptive immune responses in experimental colitis.

This thesis has an introductory chapter and the following chapters are divided into two parts. Chapter 1 is a general introduction and gives an overview of genetic, microbiological, and immunological aspects of the pathogenesis of IBD. Genetic susceptibility is required to develop IBD. However, animal models of experimental colitis have shown that intestinal bacteria are also required for the induction and perpetuation of disease. This leads to the current hypothesis that IBD is the consequence of overly aggressive immune responses to luminal commensal bacteria in the genetically susceptible host. Furthermore, chapter 1 describes treatment strategies that can alter the microflora, thereby preventing experimental colitis. These strategies include eliminating specific bacteria (antibiotics), administration of protective bacteria (probiotics), and stimulating the growth of endogenous protective bacteria (prebiotics). Innate immune responses and their intracellular pathways are discussed in chapter 2. Innate immunity is crucial as a first line of defense against pathogens. Its importance lies in the fact that innate immune responses can direct adaptive immunity.

Part I describes prevention and treatment of colitis that develops in HLA-B27 transgenic rats and IL-$10^{-/-}$ mice. Antibiotics, probiotics, and prebiotics, were used in order to manipulate the intestinal microflora in chapters 3-5, and mechanisms of attenuation of colitis were studied.

Chapter 3 gives the results of an investigation on whether broad or selective antibiotics affect onset and progression of colitis in various regions of IL-$10^{-/-}$ mice. All antibiotics (partially) prevented inflammation in the cecum and colon. However, in the treatment study ciprofloxacin and vancomycin-imipenem decreased cecal inflammation and reduced luminal *E. coli* and *E. faecalis* concentrations. In contrast, metronidazole and vancomycin-imipenem reduced colonic injury and eliminated anaerobic bacteria including *Bacteroides* spp. Thus, ciprofloxacin was most effective in the treatment of cecal inflammation; metronidazole preferentially treats the colon, whereas vancomycin-imipenem definitively treats both regions. These results suggested that subsets of aerobic or anaerobic bacteria show regional
differences in their capacity to mediate experimental colitis in IL-$10^{-/-}$ mice.

Chapter 4 contains the results of an investigation concerning the prevention of colitis with probiotic *Lactobacillus GG* in HLA-B27 transgenic rats monoassociated with *B. vulgatus*. We determined if *Lactobacillus GG* or *Lactobacillus plantarum* could treat established colitis in specific pathogen-free HLA-B27 transgenic rats and prevent recurrent disease after antibiotics were stopped. *Lactobacillus GG* did not prevent colitis in *B. vulgatus*-coassociated transgenic rats nor did it treat established disease in specific pathogen-free rats. However, *Lactobacillus GG* but not *Lactobacillus plantarum* prevented colitis relapse in antibiotic-treated rats. These studies suggested that antibiotics and probiotic agents provide synergistic therapeutic effects, perhaps mediated by altered immunomodulation with selective activity of different *Lactobacillus* species.

The aim of the study described in chapter 5 was to investigate if oral prebiotics can increase the amount of luminal probiotic bacteria and can prevent colitis in this model. We found that the prebiotic combination inulin/oligofructose partially prevented colitis in HLA-B27

transgenic rats, which correlated with altered intestinal microflora as shown by significant increases in cecal and colonic *Lactobacillus* and *Bifidobacterium* populations after prebiotic treatment compared to water-treated transgenic rats. We also observed decreased mucosal proinflammatory cytokines and increased cecal TGF-β. These results show promise for prebiotics as maintenance therapy for chronic inflammatory bowel diseases.

In Part II, bacterial-induced innate and adaptive immune responses in HLA-B27 transgenic rats and IL-$10^{-/-}$ mice were assessed. Immune cells were stimulated with bacterial products and cytokine profiles, effects of cell transfers, and intracellular pathways were studied.

Chapter 6 describes the results of a study of host-dependent immune responses to commensal cecal bacteria. We showed that in unfractionated transgenic mesenteric lymph node cells stimulated with cecal bacterial lysates, the pro- versus anti-inflammatory ratio is significantly higher compared to non-transgenic cells. Furthermore, T cell depletion abolished IFN-γ and decreased IL-12 production and conversely, neither IL-10 nor TGF-β was produced in cultures of B cell-depleted mesenteric lymph node cells. Interestingly, IL-10 and TGF-β, but not IFN-γ, IL-12, and TNF were produced by mesenteric lymph node cells from germ-free transgenic rats. These results indicate that the colitis that developed in specific pathogen-free HLA-B27 transgenic rats was accompanied by activation of IFN-γ-producing $CD4^+$ T cells that responded to commensal bacteria. However, B cell-derived cytokine production in response to components of commensal intestinal microorganisms occurred in the absence of intestinal inflammation.

The aim of the study reported in chapter 7 was to investigate the ability to induce colitis by monoassociation of HLA-B27 transgenic *rnu/rnu* rats with *B. vulgatus*, compared to their T cells-containing *rnu/+* littermates. We showed that *rnu/+* rats that contain T cells, and *rnu/rnu* transgenic rats receiving $CD4^+$ cells from *B. vulgatus nu/+* transgenic donor rats, developed colitis, whereas *rnu/rnu* rats without T cells remained disease-free. Taken together, these data indicate that T cells are required, and $CD4^+$ cells are sufficient, for the development of colitis in HLA-B27 transgenic rats monoassociated with the non-pathogenic bacterial strain *B. vulgatus*.

The study presented in chapter 8 was designed to assess the relative contribution of antigen-presenting cells versus T cells in the development of inflammation. HLA-B27 transgenic *rnu/rnu* recipients receiving either non-transgenic or transgenic mesenteric lymph node cells including T cells developed more severe intestinal inflammation compared to *rnu/rnu* non-transgenic recipients receiving either transgenic or non-transgenic cells. Interestingly, donor transgenic $CD4^+$ cells proliferated and remained at detectable levels eight weeks after adoptive transfer into *rnu/rnu* transgenic recipients but not after transfer into non-transgenic recipients. Therefore, we concluded that T cells from either non-transgenic or transgenic donors induce colitis in *rnu/rnu* transgenic but not in non-transgenic recipient rats, suggesting that activation of T cells by antigen-presenting cells expressing HLA-B27 is pivotal to the pathogenesis of colitis in this model.

The intracellular mechanisms of the dysregulated IL-12 responses in the absence of IL-10 in IL-$10^{-/-}$ mice are poorly understood. Chapter 9 reports the results of a study of the intracellular pathways NF-κB and STAT3 in LPS-induced IL-12p 40 gene expression in BMDC isolated from wild-type and IL-$10^{-/-}$ mice. Surprisingly, LPS-induced NF-κB signaling was similar in IL-$10^{-/-}$ and wild-type BMDC. Chromatin immunoprecipitation demonstrated sustained NF-κB binding to the IL-12 p40 promoter in IL-$10^{-/-}$ but not wild-type BMDC. Adenoviral gene delivery of a constitutively active STAT3 blocked LPS-induced IL-12p 40 gene expression, and NF-κB recruitment to the IL-12 p40 promoter. Therefore, we concluded that dysregulated LPS-induced

IL-12 p40 gene expression in IL-10$^{-/-}$ mice is due to enhanced and sustained NF-κB recruitment to the IL-12 p40 promoter in the absence of activated STAT3.

In conclusion, this thesis emphasized the importance of bacteria and their products in the induction and perpetuation of experimental colitis. Further research is necessary to elucidate the underlying mechanisms that mediate the pathogenic and protective effects of intestinal bacteria in experimental colitis and IBD.

Samenvatting

Mechanismen van bacterie-geïnduceerde experimentele colitis

Chronische inflammatoire darmziekten (IBD) omvatten colitis ulcerosa en de ziekte van Crohn, en hebben een grote invloed op de kwaliteit van leven van patiënten. Onze kennis van deze chronische ziekten is beperkt, hetzelfde geldt voor de huidige behandelings-strategieën. Om de oorzaken van IBD beter te begrijpen besteedt dit proefschrift aandacht aan (1) de verbetering van bacterie-geïnduceerde experimentele colitis door middel van manipulatie van de darmflora, en (2) de regulatie van bacterie-geïnduceerde verworven en aangeboren immuun reacties in experimentele colitis.

Dit proefschrift begint met een algemene introductie en de daaropvolgende hoofdstukken zijn opgedeeld in twee delen. Hoofdstuk 1 geeft een overzicht van de genetische, immunologische, en microbiologische aspecten bij de pathogenese van IBD. Genetische aanleg is een vereiste voor het ontwikkelen van IBD. Onderzoek van experimentele colitis in diermodellen heeft echter aangetoond dat ook darmbacteriën vereist zijn voor het ontwikkelen van colitis. Deze gegevens hebben geleid tot de huidige hypothese dat IBD het gevolg is van een over-agressieve immuunreactie gericht tegen de darmflora van een genetisch gevoelige gastheer. Ook zijn in hoofdstuk 1 behandelingsstrategieën besproken die de darmflora kunnen veranderen om zodoende experimentele colitis te voorkomen. Voorbeelden hiervan zijn het elimineren van specifieke bacteriën (antibiotica), de toename van de concentratie beschermende bacteriën (probiotica) en de stimulatie van de groei van endogene beschermende bacteriën (prebiotica). In hoofdstuk 2 wordt separaat nog wat dieper ingegaan op de aangeboren immuun reacties en de bijbehorende onderliggende intracellulaire mechanismen. Aangeboren immuniteit is cruciaal voor de eerstelijns afweer tegen pathogenen. Tevens is aangeboren immuniteit belangrijk voor de sturing van verworven immuniteit.

Deel I beschrijft de preventie en behandeling van experimentele colitis in HLA-B27 transgene ratten en IL-10$^{-/-}$ muizen. In de hoofdstukken 3 t/m 5 werden antibiotica, probiotica, en prebiotica toegepast om de darmflora te manipuleren, en tevens werden mechanismen van bescherming tegen darmontsteking onderzocht.

In hoofdstuk 3 hebben wij onderzocht of breed- en smalspectrum antibiotica ontsteking in de diverse regionen van het colon van IL-10$^{-/-}$ muizen zou kunnen beïnvloeden. Deze studie toonde aan dat alle antibiotica ontsteking in zowel het caecum als het colon (gedeeltelijk) konden voorkomen. In het behandelingsprotocol bleken ciprofloxacine en vancomycine-imipenem echter specifiek de ontsteking te remmen in het caecum en tevens de luminale concentraties van *E. coli* and *E. faecalis* te reduceren. Daarnaast bleken metronidazol en vancomycine-imipenem de ontsteking in het colon te reduceren en in staat anaerobe bacteriën inclusief *Bacteroides* stammen te elimineren. Concluderend blijkt dat ciprofloxacine het meest effectief is in het caecum,

metronidazol in het colon, en vancomycine-imipenem is effectief in beide regionen. Deze resultaten suggereren dat subgroepen van aerobe en anaerobe bacteriën regionale verschillen vertonen wat betreft de capaciteit van het onderhouden van colitis in IL-$10^{-/-}$ muizen.

In hoofdstuk 4 is onderzocht of de probiotische bacterie *Lactobacillus GG* colitis kan voorkomen in HLA-B27 transgene ratten in monoassociatie met *B. vulgatus*. Tevens werd onderzocht of *Lactobacillus GG* danwel *Lactobacillus plantarum* fulminante colitis in specifiek pathogeen-vrije transgene ratten kon behandelen en een recidief kon voorkomen na het stoppen van antibiotische behandeling. *Lactobacillus GG* kon colitis niet voorkomen in *B. vulgatus*-co-geassocieerde transgene ratten, en was evenmin in staat om fulminante colitis te behandelen. *Lactobacillus GG* kon echter een recidief colitis voorkomen in ratten behandeld met antibiotica, dit gold niet voor *Lactobacillus plantarum*. Deze resultaten suggereren dat antibiotica en probiotica mogelijk synergistische therapeutische effecten hebben, mogelijk ten gevolge van een immunomodulatoire werking met een selectieve activiteit van de verschillende *Lactobacillus* soorten.

Het doel van de studie beschreven in hoofdstuk 5 was te onderzoeken of orale prebiotica de luminale concentraties van probiotische bacteriën kunnen verhogen en colitis kunnen voorkomen. De prebiotische combinatie inuline/oligofructose voorkwam gedeeltelijk colitis in HLA-B27 transgene ratten. Dit correleerde met een veranderde darmflora, zoals aangetoond middels significante toename van luminale concentraties van *Lactobacillus* en *Bifidobacterium* soorten, vergeleken met de water controle groep. Tevens vonden wij een afname van intestinale pro-inflammatoire cytokinen en een toename van caecale TGF-β. Deze resultaten bevestigen de veelbelovende mogelijkheden van prebiotica als potentiële onderhoudstherapie bij patiënten met IBD.

In Deel II zijn bacterie-geïnduceerde aangeboren en verworven immuun reacties in HLA-B27 transgene ratten en IL-$10^{-/-}$ muizen beschreven. Daartoe werden immuun cellen gestimuleerd met bacteriële producten en vervolgens werden cytokine profielen, de effecten van cel transfers, en intracellulaire signaaltransductie onderzocht.

In hoofdstuk 6 is aangetoond dat wanneer ongefractioneerde cellen van mesenteriale lymfeklieren uit HLA-B27 transgene ratten gestimuleerd worden met caecale bacteriële lysaten een grote toename van de pro/anti-inflammatoire cytokine ratio gezien wordt in vergelijking met non-transgene cellen. De IFN-γ reacties verdwenen en de IL-12 productie minimaliseerde na depletie van T cellen, terwijl B cel depletie de IL-10 en TGF-β productie elimineerde. Tenslotte produceerden mesenteriale lymfeklier cellen van kiemvrije ratten wel IL-10 en TGF-β, maar geen IFN-γ, IL-12, en TNF. Deze resultaten suggereren dat colitis in HLA-B27 transgene ratten samenhangt met de activatie van IFN-γ producerende $CD4^+$ T cellen die reageren op commensale bacteriën. Daarentegen produceren B cellen anti-inflammatoire cytokinen na stimulatie door componenten van intestinale micro-organismen, ook in afwezigheid van ontsteking.

Het doel van hoofdstuk 7 was te onderzoeken of *B. vulgatus* colitis kan veroorzaken in monogeassocieerde HLA-B27 transgene *rnu/rnu* ratten en *rnu/+* ratten die T cellen bevatten. In dit onderzoek hebben wij aangetoond dat *rnu/+* ratten met normale T cel ontwikkeling, en ook *rnu/rnu* transgene ratten na transfer van $CD4^+$ cellen van *B. vulgatus rnu/+* transgene donor ratten, colitis ontwikkelden. Echter, *rnu/rnu* ratten zonder T cellen bleven ziekte-vrij. Samenvattend blijkt uit deze data dat T cellen vereist zijn en $CD4^+$ T cellen voldoende voor het mediëren van colitis door de non-pathogene bacterie *B. vulgatus* in HLA-B27 transgene ratten.

De studie beschreven in hoofdstuk 8 werd ontworpen om het relatieve aandeel van antigeen-presenterende cellen en T cellen in de ontwikkeling van colitis te bepalen. HLA-B27

transgene *rnu/rnu* ontvangers van non-transgene danwel transgene mesenteriale lymfeklier cellen inclusief T cellen ontwikkelden ernstige colitis vergeleken met *rnu/rnu* non-transgene ontvangers van non-transgene danwel transgene cellen. Opvallend genoeg bleken donor transgene $CD4^+$ cellen te prolifereren in transgene ontvangers en nog detecteerbaar te zijn na acht weken, dit gold niet voor non-transgene ontvangers. Concluderend induceren T cellen van non-transgene en transgene donoren colitis in *rnu/rnu* transgene maar niet in non-transgene ontvangers. Dit suggereert dat activatie van T cellen door antigeen-presenterende cellen die HLA-B27 tot expressie brengen cruciaal is in de pathogenese van colitis in dit model.

De intracellulaire mechanismen van ontregelde IL-12 reacties in de afwezigheid van IL-10 in $IL\text{-}10^{-/-}$ muizen zijn onbekend. Daarom hebben wij in hoofdstuk 9 de intracellulaire signaaltransductie cascades voor NF-κB en STAT3 onderzocht in relatie tot LPS-geinduceerde IL-12 p40 gen expressie in dendritische cellen uit het beenmerg (BMDC) van wild-type en $IL\text{-}10^{-/-}$ muizen. Verrassend genoeg bleek er geen verschil te zijn tussen LPS-geinduceerde NF-κB signaaltransductie in $IL\text{-}10^{-/-}$ en wild-type BMDC. Chromatine immunoprecipitatie analyse vertoonde een blijvende binding van NF-κB aan de IL-12 p40 promotor in $IL\text{-}10^{-/-}$ maar niet in wild-type BMDC. Een kunstmatige continue activatie van STAT3 middels een adenovirale gen transfer blokkeerde de LPS-geinduceerde IL-12 p40 gen expressie, en tevens de binding van NF-κB aan IL-12 p40 promotor. Samenvattend is de ontregelde LPS-geinduceerde IL-12 p40 gen expressie in $IL\text{-}10^{-/-}$ muizen het gevolg van langdurig toegenomen binding van NF-κB aan de IL-12 p40 promotor bij het ontbreken van geactiveerd STAT3.

Concluderend benadrukken de resultaten beschreven in dit proefschrift de cruciale rol voor bacteriën en hun producten voor het ontstaan en onderhouden van experimentele colitis. Toekomstig onderzoek is nodig om de onderliggende mechanismen die de de pathogene maar ook beschermende werking van darmbacteriën in experimentele colitis en IBD mediëren te verklaren.

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Acknowledgements

Dankwoord

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Curriculum Vitae

Curriculum Vitae

Frank Hoentjen werd op 23 oktober 1975 geboren te Bennekom. Hij haalde zijn VWO-diploma aan het Van Lingen College te Arnhem in 1994. In datzelfde jaar begon hij aan de studie geneeskunde aan de Katholieke Universiteit Nijmegen, hetgeen in 1999 resulteerde in het doctoraal examen met het predikaat cum laude. Gedurende deze periode volgde Frank een wetenschappelijke stage van 1996 tot en met 1999 aan de afdeling Maag-Darm-Leverziekten van het Academisch Ziekenhuis St. Radboud te Nijmegen, gericht op de regulatie van motoriek en secretie van de tractus digestivus. Voor dit werk ontving hij in 2000 de Studentenprijs van de Nederlandse Vereniging voor Gastroenterologie. Tijdens zijn co-schappen volgde hij een wetenschappelijke stage aan de University of North Carolina at Chapel Hill, USA, met als aandachtsgebied de rol van bacteriën in de pathogenese van experimentele colitis. Voor dit onderzoek ontving hij de Student's Award van de American Gastroenterological Association. Tevens volgde hij een klinische tropenstage aan de Gadjah Mada University te Yogyakarta, en de University of Indonesia te Jakarta, Indonesië. Het artsexamen werd behaald in 2002, en vervolgens werd Frank aangesteld als assistent-geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) aan de Vrije Universiteit Amsterdam. Als onderdeel daarvan begon hij zijn promotie onderzoek in september 2002, verricht aan de University of North Carolina at Chapel Hill, USA. Voor dit werk ontving hij onderscheidingen van de Gastroenterology Research Group and American Gastroenterological Association, en de European Gastroenterology Federation. Op dit moment werkt Frank in het St. Lucas Andreas Ziekenhuis te Amsterdam in het kader van zijn opleiding tot maag-darm-leverarts, naar verwachting af te ronden in 2009.

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