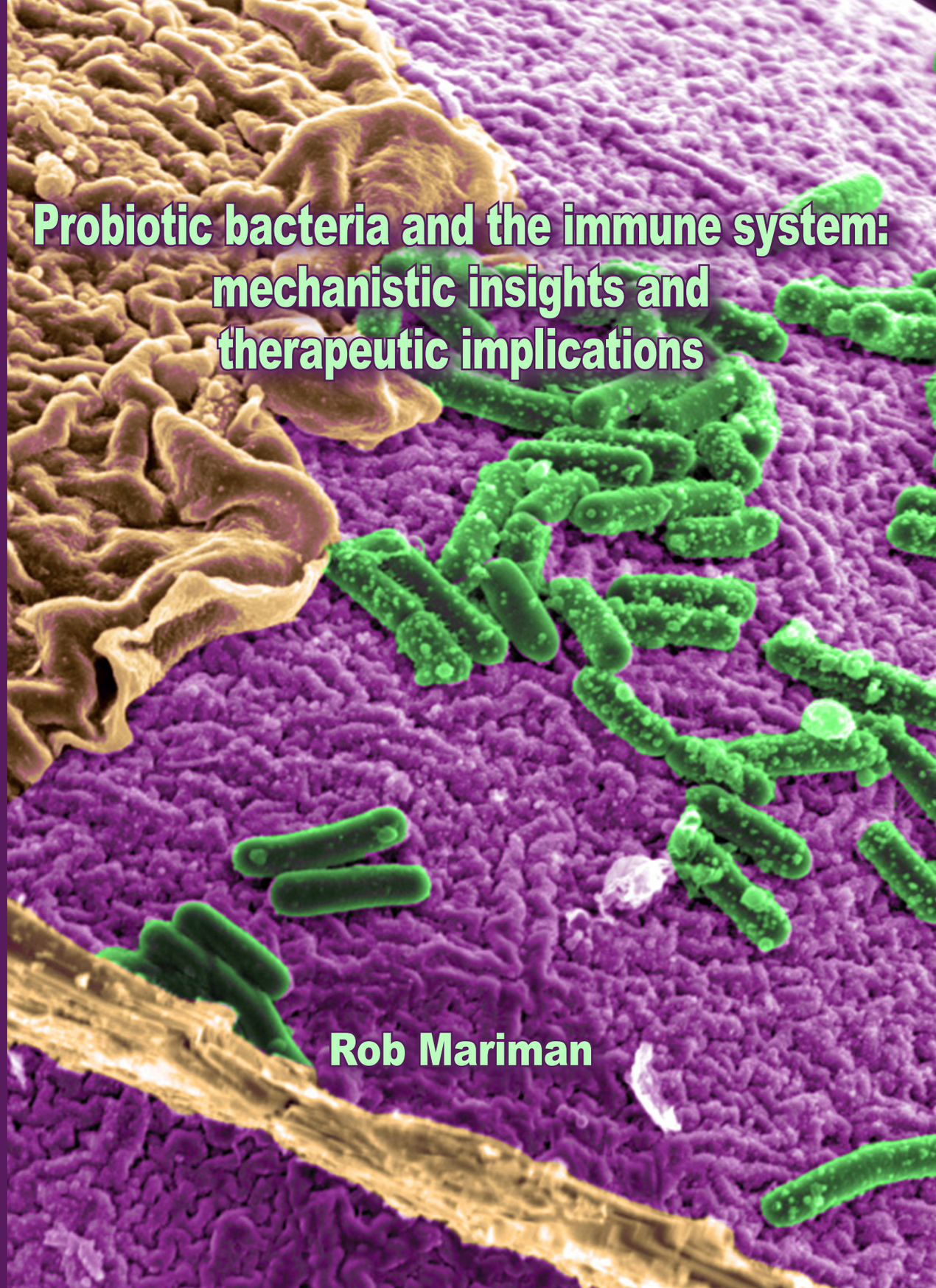
Probiotic bacteria and the immune system: mechanistic insights and therapeutic implications

Rob Mariman



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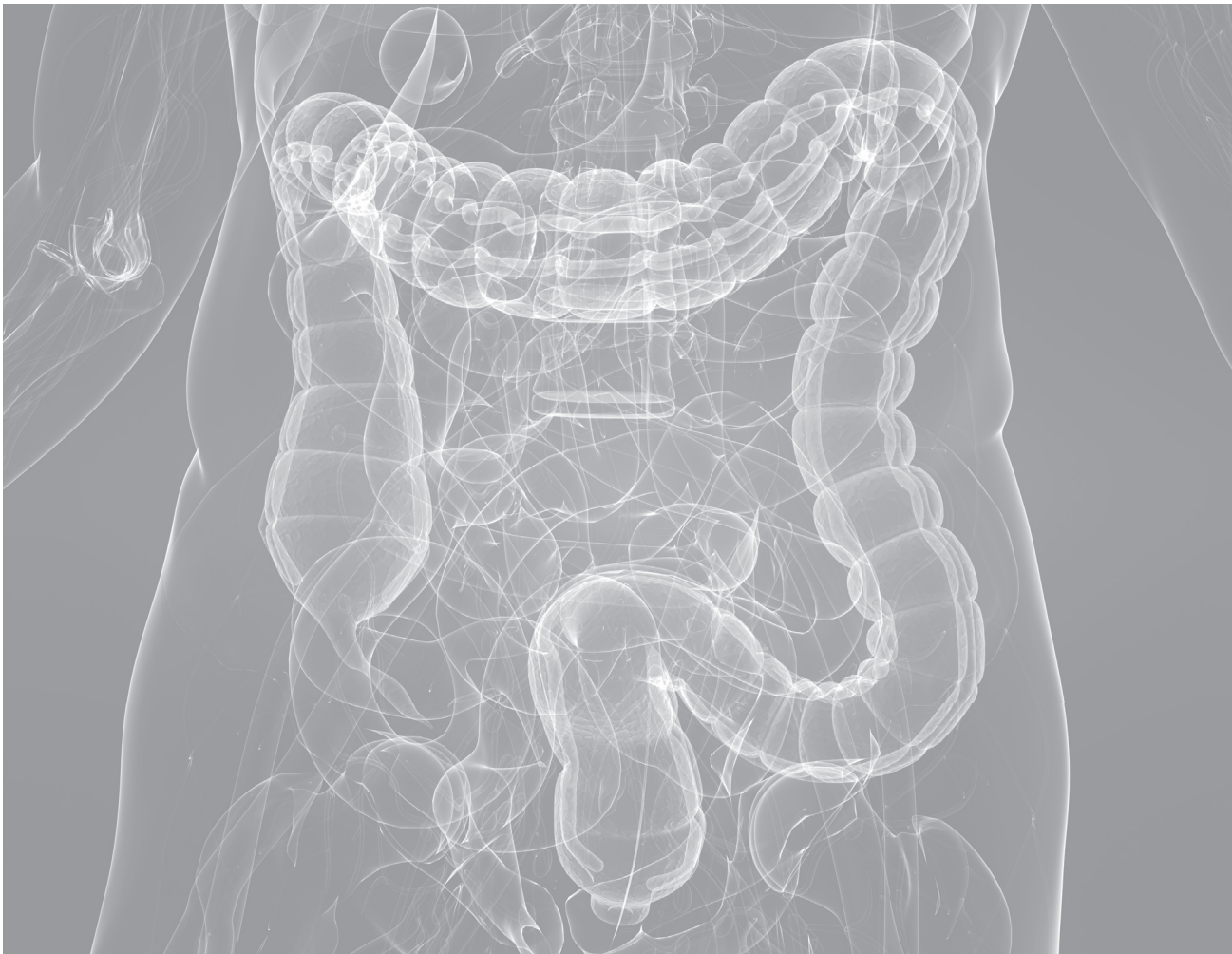
Chapter 1

General introduction



1.1 Inflammatory Bowel Disease

The two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic relapsing intestinal inflammation [1]. This life-long disorder may occur at any time, although 80% of the cases are diagnosed in early adult life. The incidence and prevalence of IBD are increasing with time and affects nowadays approximately 3.6 million people in the USA and Europe [2]. UC is limited to the colon, and microscopical features include superficial inflammation infiltration in the mucosa and submucosa with cryptitis and crypt abscesses. By contrast, CD involves any part of the GI-tract in a non-continuous fashion. Histologically, CD is characterized by thickened submucosa, transmural inflammation and tissue ulcerations [1]. The exact etiology of IBD is largely unknown but genetic, environmental and intestinal microbial factors have been reported to play a role [3]. Moreover, these inflammatory diseases result in an inadequate balance between T regulator (Treg) and T effector responses, in particular T_h1/T_h17 cells in CD and T_h2 cells in UC [4].

1.1.2 IBD genetics

Genome wide association studies (GWAS) have identified 163 genetic risk loci underlying IBD susceptibility [5,6]. 30% of these genetic loci are shared between CD and UC, suggesting similar genetic predispositions and pathological pathways. IBD susceptibility genes are linked to pathways/processes involved in intestinal homeostasis, including barrier function, innate immune function, reactive oxygen species generation, endoplasmic reticulum stress and metabolic pathways associated with cellular homeostasis [7]. Susceptibility genes related to the innate immune response include a repertoire of innate immune receptors like NLRP3, RIG-1, TLR3, TLR5 and NOD2 [8-11]. Mutations in NOD2 impairs activation of NF-κB in response to peptidoglycan through the recognition of muramyl dipeptide(MDP), thereby modulating both innate and adaptive immune responses [12]. Also expression profiles of adaptor molecules involved in TLR/NOD signaling pathways are affected in IBD patients e.g. CARD9. Depending on the stimulus, CARD9 interacts with distinct signaling complexes and activates different pathways to modulate cytokine environments properly [4]. Defective CARD9 function results in failure to promote an adequate Th17 immune response against intracellular pathogens [13]. Other genetic associations in the innate arm of the immune system comprise the autophagy related genes ATG16L1 and IRGM [14,15]. Autophagy represents the self-digestion of organelles and ingested extracellular bacteria and other types of pathogens via lysosomal degradation, and defects are implicated in the immuno pathogenesis of IBD [16,17]. GWAS also identified susceptibility genes related to the adaptive arm of the immune system, and includes TNFR15, IL-2, JAK2, TYK2, STAT3, CCR6, and IL-23R [18]. IL-23R is a key differentiation feature of $CD4^+T_h17$ cells that are critical in mediating

antimicrobial defenses [19]. Balancing between IL-17/Treg responses is critical for optimizing defenses to microbes while simultaneously avoiding chronic tissue inflammation. Another IBD linked locus involves genes linked to anti-inflammatory responses like IL-10, IL-27 and IL-10Rα, demonstrating the importance of a proper balance of pro and anti-inflammatory regulators. However, the heterogeneity of IBD and the low disease penetrance in individuals carrying disease-susceptibility alleles suggest that several host and environmental factors interact to cause IBD [20]. Therefore, the so-called multi-hit hypothesis was proposed, were the induction and perpetuation of chronic intestinal pathology requires at least two triggers such as disturbed mucosal barrier and impaired immune regulation in the gut (Figure. 1).

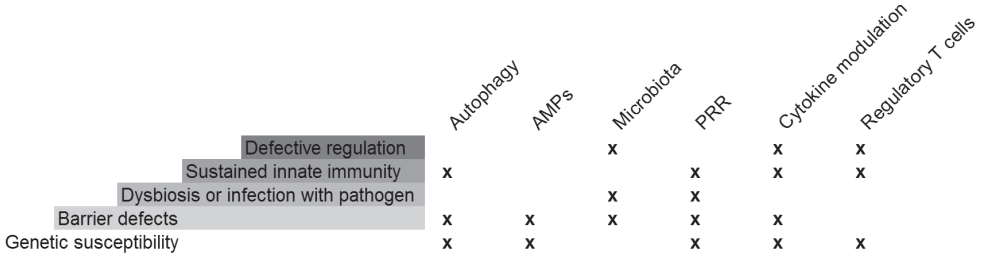


Figure 1. A multi-hit model of IBD pathogenesis. The induction and perpetuation of chronic intestinal pathology may require the convergence of many abnormalities that affect several overlapping layers of immune homeostasis in the intestine (adapted from Maloy & Powrie, *Nature* 2011: 298-306)

1.1.3 Experimental models for IBD

To study the pathogenesis of IBD, many animal models have been developed reflecting different aspects of disease, but no model completely recapitulates the characteristics of human disease [21]. Most IBD models result from exogenous manipulation; they can be categorized based on induction by chemicals, pathogens, immune cell transfer, or gene targeting [22].

Spontaneous development of colitis occurs in mice with particular genetic defects produced by either gene targeting or the introduction of a transgene, including IL-10 knockout mice and $TNF^{(\Delta are)}$ transgenic mice. Also transfer of $CD45RB^{+}$ T cells to immune-deficient mice results in the development of colitis [23]. One notable disadvantage of these models is that they are established in immune-compromised mice in contrast to chemically induced models.

Most widely chemical induced models used for preclinical efficacy studies are dextran sulfate sodium induced (DSS) and trinitrobenzene sulfonic acid (TNBS) colitis. In the DSS model, the epithelial layer is disrupted resulting in increased permeability and translocation of intestinal bacteria. After induction mice develop colonic inflammation, which do not require

adaptive immune cells. Therefore, this acute model is suitable to study the effect of new compounds on epithelial cell barrier. The TNBS colitis model is a hapten-induced colitis model, based on the intrarectal administration of TNBS in ethanol. Inflammation is induced by a two-step process; first the ethanol impairs the epithelial barrier. Subsequently, TNBS haptenizes autologous antigens and microbial proteins rendering them immunogenic to the host [24]. Notably, all models for intestinal inflammation are driven by the resident microbiota, since inflammation fails to develop in germ-free mice [25,26].

1.2.1 Crosstalk between the intestinal microbiota and the immune system

As indicated in the previous paragraph, disturbance of the dynamic balance between microbes, particularly commensal flora, and host defensive responses at the mucosal frontier plays a pivotal role in the initiation and pathogenesis of chronic IBD. Immediately after birth, the mammalian gastro-intestinal tract (GI-tract) is colonized by a dynamic community of microorganisms, referred to as the gut microbiota [27]. The human microbiota consists of 10^{14}microbial cells, with highest densities in the distal colon with up to 10^{11}-10^{12} organisms per gram of luminal content [28]. This microbiota community establishes a symbiotic relationship with its host. They supply essential nutrients and metabolize indigestible compounds, and defend against the colonization by pathogenic bacteria, while occupying a protected nutrient rich environment [29]. The intestinal immune system, which is exposed to an enormous bacterial load, has a critical role in limiting tissue invasion by these resident bacteria. Host-microbiota homeostasis is achieved by various immunological barriers. The first barrier is the limitation in exposure of the epithelial layer to luminal content by secretion of mucus, anti-microbial peptides and IgA, thereby creating a physical barrier. Bacteria that penetrate this semi-sterile layer and breach the epithelial barrier are recognized by innate immune cells and undergo rapid phagocytosis followed by the induction of various T cell responses [30].
Interactions between microbes and the host are of critical importance in orchestrating the immune system, as shown in studies with germ-free mice. Germ-free mice are sterile mice and show defects in the development of gut associated lymphoid tissue (GALT)[31] reflected by reduced antibody production by IgA plasma cells [32], fewer Peyer's Patches (PP) and mesenteric lymph nodes(MLN) as well as impaired development of isolated lymphoid follicles [29]. In these mice, the structure and function of epithelial cells is also impaired, which is reflected by decreased rates in cell turnover and altered microvilli structures. Moreover, absence of microbiota impairs proper balance of chemokine, cytokine and defensin production by intestinal epithelial cells (IEC) and immune cells. Altogether, these defects make germ-free mice more susceptible to infection by pathogenic bacteria, viruses and parasites. Host-microbiota interactions not only shape the immune system, but in turn the immune system also shapes the composition of the microbiota. This was demonstrated in a study with TLR5

deficient mice that lack the receptor for bacterial flagellin [33]. Absence of TLR5 results in changes in the composition of the microbiota and symptoms of the metabolic syndrome.

1.2.2 Dysbiosis of the microbiota and disease

Dysbiosis refers to the imbalance in the composition of the microbiota, which includes the outgrowth of potentially pathogenic bacteria and a decrease in bacterial diversity and bacteria beneficial to the host [34]. Factors contributing to dysbiosis include host genetics, altered microbes exposure, and medical practice like vaccination and use of antibiotics. Moreover, lifestyle which includes diet, stress and hygiene could also contribute to dysbiosis. Resultant changes in host-microbiota interactions might underlie the large increase in the incidence of several immune related disorders such as atopic dermatitis, IBD, asthma, rheumatoid artitis and multiple sclerosis [35]. Data supporting this 'altered microbiota' interpretation include the correlation between asthma/allergies and use of antibiotic in industrialized countries [36], and the correlation between GI-microbiota of newborns and the development of allergic diseases [37].

In the context of intestinal disorders, an altered gut microbiota has been observed in patients with inflammatory bowel disease compared to healthy individuals [38,39]. However, no pathogenic species have been identified so far. It might be that in normal conditions commensal bacteria do not elicit an immune response but have the potential to become pathogenic only when genetic or environmental conditions are altered in the host [28]. These species are reflected to pathobionts which cause an immunological disequilibrium under conditions of dysbiosis.

1.2.3 Immune modulation by probiotics

Since the microbiota is of critical importance in shaping the intestinal immune system, modulation of the microbiota with pre-or probiotics might be a useful strategy to prevent immunological disorders. Probiotic have been defined by the WHO as "live micro-organisms which when administered in adequate amounts confer a health benefit for the host" [40]. Most probiotics are gram positive lactic acid bacteria, mainly consisting of *Lactobacillus* and *Bifidobacterium* species [41], but also the gram negative *Escherichia coli* strNissle 1917 has been shown to exert beneficial effects [42].

Several mechanisms of action have been proposed in which probiotics assert these beneficial effects. First, they can interfere with the ability of pathogens to colonize and infect the mucosa [43]. This can be achieved by either direct inhibitory effect on pathogens, by competitive exclusion, or through the influence of probiotic bacteria on the composition of the endogenous commensal flora [44]. Secondly, probiotics have been shown to stabilize and maintain the intestinal barrier. Enhancement of epithelial barrier function can be achieved through general antimicrobial effects like the secretion of mucus or defensins

[45], effects on epithelial cell survival [46], and modulation of tight-junction function [47,48]. Furthermore, probiotics may modulate enzymatic activity in the intestine, for example by inhibition of pro-carcinogenic enzymes or the induction enzymatic activity that favors food nutrition [49]. Probiotics may also directly modulate local en systemic immune responses of the host [50,51]. Direct modulation occurs after interaction of these bacteria with epithelial and (innate) immune cells in the intestinal mucosa, which contains the largest reservoir of immune cells in the body. Compared to the colon, endogenous bacteria load is relative low in the small intestine, and thereby the most likely target site for probiotics in the modulation of innate and adaptive immune responses [52].

1.3.1 Microbial sensing via pattern recognition receptors

Pattern recognition receptors (PRR) include Toll-like receptors (TLR), nucleotide binding oligomerization domain receptors (NOD-like receptors), and C-type lectin receptors [53]. These receptors recognize a variety of microbial components and allow (innate) immune cells to sense pathogens associated molecular patterns (PAMP) on bacteria, viruses, fungi and parasites, but also endogenous danger associated molecular patterns.

In the intestine, these receptors are expressed on IEC and antigen presenting cells (APC) and play a central role in governing the interface between host and microbiota [54]. Cell surface TLR recognize mainly bacterial surface products such as lipopeptides and peptidoglycan (TLR 1, -2, -6), lipopolysaccharide (LPS) – a major component of gram negative bacteria (TLR4), and flagellin (TLR5). TLR 3, -7, -8 and 9 reside in the intracellular organelles and sense microbial nucleic acids [55]. NOD-like receptors detect peptidoglycan components of the bacterial cell wall [56]. In contrast, C-type lectin receptors recognize specific carbohydrate structures on self-antigens or cell wall components of pathogens. Their main function is to internalize antigens for degradation in lysosomal compartments to enhance antigen presentation [57,58].

Despite various physical barriers, PRR in the gut are exposed to an enormous microbial load. To prevent excessive activation of these receptors, the host has developed a set of redundant overlapping processes to prevent disproportionate immune activation in steady-state conditions, while maintaining the ability to be activated upon infection [59]. These strategies to avoid excessive activation of immune cells include the expression of negative regulators of TLR signaling upon activation, like IL-10, Tollip and IL-1Ra [60,61] or the co-stimulation of receptors like DC-SIGN that interfere with TLR signaling [62]. Selective expression of PRR on innate immune cells also prevents aberrant receptor activation. Under homeostatic conditions for example, TLR2 and TLR4 and several co-factors required for signal transduction are barely expressed, but these receptors become up-regulated upon inflammation [63]. Selective TLR5 expression on the basal site of IEC is also such

a mechanism in which the host only responds to flagellin-bearing bacteria that invade the epithelium layer[64].

1.3.2 Dendritic Cells in the gastro-intestinal tract

Dendritic cells (DC) are professional bone marrow derived antigen presenting cells (APC) that migrate from peripheral tissues to secondary lymphoid organs for antigen presentation to T cells [65]. In the gut, DC continuously migrate from the mucosa to the MLN, thereby inducing tolerance to "self" and food antigens or protective immunity [66]. Antigen-uptake by mucosal DC can be facilitated by microfold cells, that are specialized epithelial cells found on the surface of the follicle-associated epithelium (FAE) that transfer antigenic material to DC beneath the epithelia layer [67]. The process of antigen-uptake can also, although less efficient, be facilitated by epithelial cells. Moreover, DC can also sample antigens directly with dendrites that stick through the epithelium into the lumen [68]. Since the intestinal mucosa is an important entry site for pathogens, but also home to a large and diverse commensal microflora, intestinal APC have to discriminate between friend and foe. The mechanisms by which various intestinal DC subsets respond to environmental triggers, thereby shaping local immune responses will be discussed in the following paragraphs.

1.3.3 Intestinal DC subsets

In the gut, APC are found in the lamina propria (LP), isolated lymphoid follicles, PP, and the MLN. APC that survey the intestinal mucosa comprise several different phenotypes dependent on their anatomical localization. This APC pool includes various subpopulations of macrophages, conventional $CD11c^{hi}$ DC, and plasmacytoid DC (pDC). All subtypes have been adapted to the intestinal environment to prevent the generation of destructive inflammatory responses, but ensure protection against enteric pathogens [69]. Classification of intestinal DC subsets is often based on the expression of cell surface receptors.

DC found in the PP, which are organized lymphoid structures in the small intestine, are mainly consist of $CD11c^{hi}CD11b^{+}CD8\alpha^{-}$, $CD11c^{hi}CD11b^{+}CD8\alpha^{+}$ and $CD11c^{hi}CD11b^{-}CD8\alpha^{-}$ conventional DC [70]. These DC modulate T-cell skewing, where $CD11b^{+}$ DC are linked to Th2 polarization contrary to $CD8\alpha^{+}$DC that induce Th1 responses.

Mononuclear phagocytic cells located in the LP can be divided into different subgroups with functionally different properties and can be distinguished by the expression of surface receptors. Main subtypes include $CX3CR1^{-}CD68^{-}CD103^{+}$ DCs and a population of classical macrophages that are $CX3CR1^{+}CD68^{+}F4/80^{+}$ [71,72]. $CD103^{+}$DC subset express 2,3-dioxgenase (IDO) and drive regulatory T-cell (Treg) development in the MLN via TGF-β and retonic acid [73,74]. By contrast mouse CX_3CR1^{+}phagocytes have been shown to drive the development of naïve T cells to Th1 and Th17 cells [75,76], presumably via a flagellin or ATP-dependent mechanism [77] as shown in figure 2.

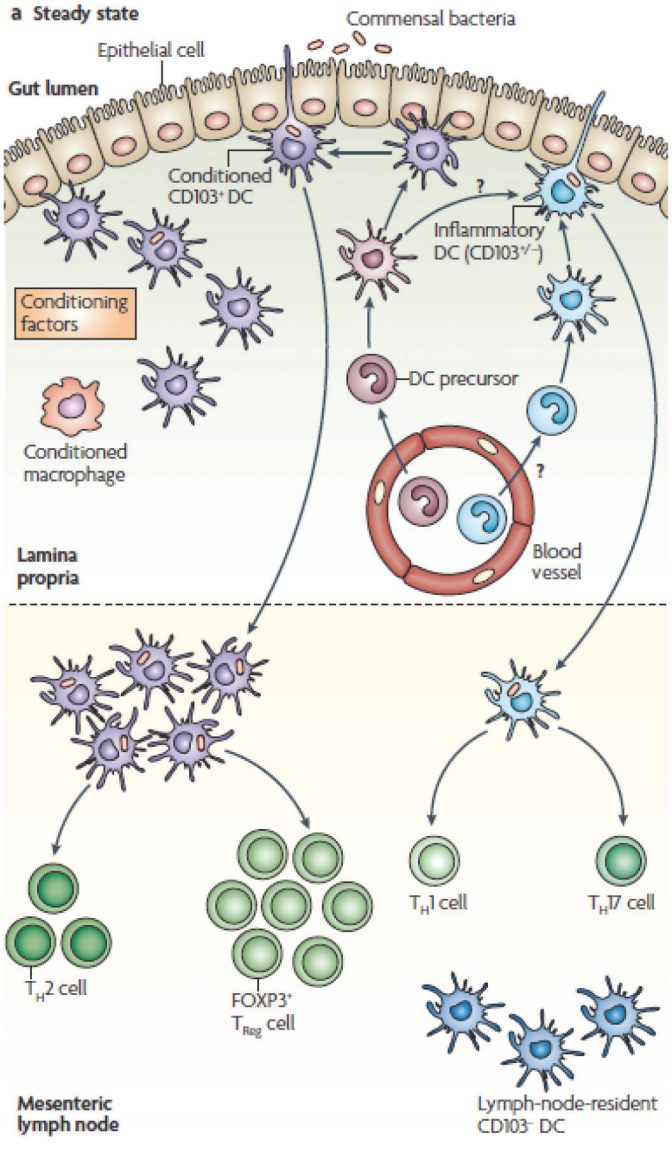


Figure 2. Schematic overview of DC resident in the intestinal mucosa. In response to commensal microflora or self-antigens DC migrate to the MLN and drive T cell differentiation. (Adapted from Coombes & Powrie, *Nature Immunology*. 2007: 435-446)

1.4 Shaping intestinal immune cell populations by selective chemokine expression

As described in the previous section, mucosal DC can elicit effector responses that lead to the secretion of distinct patterns of cytokines thereby dictating the skewing of naïve Th cells to effector T cells. Likewise, small chemo-attractive cytokines defined as chemokines, play a critical role in directing the balance between physiological and pathophysiological inflammation in health and IBD [78]. Chemokines secreted by IEC and APC in the intestinal mucosa play an important role in shaping immune cell populations, - including B, -T lymphocytes, neutrophils and mast cells - by targeting specific receptors on these cells [79]. In conditions of homeostasis, a subfamily of so called homeostatic or constitutively expressed chemokines play a key immune-surveillance role in lymphocyte and DC trafficking between primary and secondary lymphoid tissue [80].These chemokines include CCL25 and CCL28 which are produced by epithelial cells of the small intestine and colon, respectively [81]. Also CCL9, CCL20 and CXCL16 produced in the FAE are defined as homeostatic since they contribute to the organization and shaping of this tissue in steady state conditions.
Another class of chemokines comprises those that are induced upon inflammation, which lead to the recruitment of leukocytes, including DC, to the inflamed tissue. Active inflamed tissue of inflammatory bowel disease patients showed increased expression levels of CXCL8, CCL2, CCL3, CCL4 CCL11 and CCL24 [82], which resulted in increased trafficking and localization of monocytes, NK-cells and T lymphocytes in the mucosa. Since leukocyte infiltration into the intestine is a fundamental event in disease development and progression, modulation of chemokine expression might be a suitable strategy to dampen inflammation.

1.5 Aim of this thesis

This thesis aimed to provide insight into the role of microbiota-host interactions in the regulation of mucosal and systemic immunity in the context of IBD. Regulation of microbiota composition (e.g. by probiotics and prebiotics) offers the possibility to modulate immune responses and contribute to the prevention and treatment of (autoimmune) - diseases.
By evaluating immune modulation capacities of probiotics with genome-wide gene expression profiling in both *in vivo* and *in vitro* models, novel mechanisms were identified in which probiotic bacteria modulate immune responses under conditions of homeostasis and inflammation. These new insights will allow more rational selection and validation of probiotic usage in a variety of clinical conditions.
Chapter 2 describes the characteristics of the recurrent TNBS colitis model in BALB/c mice. Processes underlying the development of pathology in this model were identified by temporal

gene expression profiling of the colon. It is demonstrated that the recurrent TNBS-induced colitis model combines histopathological features with aspects of active inflammation and chronic processes of cellular infiltration, angiogenesis and tissue remodeling, which are all relevant to human IBD. Furthermore, the sensitivity of the model for immunosuppression by local treatment with budesonide was evaluated. In **chapter 3** this model was used to evaluate the efficacy of two probiotic preparations – *L.plantarum* and VSL#3–in dampening intestinal inflammation. To this end, genome wide screening of inflamed colonic tissue was applied in order to identify processes and pathways that are modulated by probiotics.

The interaction between the intestinal microbiota and the gut-associated mucosal immune system is considered to play a key role in inflammatory diseases in the gut [83], as well as at peripheral sites like the skin [84][85,86]. In **chapter 4**, the association between gut inflammation and atopic dermatitis was studied in human ApoC1$^{+/+}$ transgenic mice that display features of atopic dermatitis. Furthermore, the potential suppressive effect of probiotic bacteria was evaluated in this model. To further substantiate the observed immune modulating effects of probiotic bacteria in mouse models, the modulation of bone-marrow derived dendritic cells was studied. To account for the genetic variation among populations, DC derived from mice with different genetic backgrounds were studied. Emphasis was put on the modulation of TLR and their downstream signaling pathway, since immune-modulatory effects of commensal and probiotic bacteria may rely on their interaction with these receptors. The studies described **in chapter 5** refer to the activity of VSL#3 – a mixture of 8 probiotic strains – as an approach to the complexity of different types of interaction *in vivo* as opposed to the effects of ultrapure ligands which may only interact with single TLR. To facilitate translation of observations with mouse DC to the human setting, the effects of probiotic bacteria on human DC were evaluated as well. **Chapter 6** describes the effect of probiotics on human monocyte derived DC with emphasis on the modulation of cytokine and chemokine expression, employing dedicated gene arrays and multiplex protein profiling. Furthermore, we aimed to identify pathways accounting for the anti-inflammatory effect of probiotics on DC.

The *in vivo* models employed in this thesis are likely to have compromised intestinal barrier, which could facilitate the accessibility of the probiotics to the immune cells and the lymphoid tissues. Therefore, studies in the disease state may not reflect and predict the immune-modulatory effects of probiotic bacteria in healthy individuals. This issue was addressed in **chapter 7**, where the immune-modulatory effects of probiotic bacteria in healthy, non-diseased mice were evaluated. Finally, **Chapter 8** and **Chapter 9** (in Dutch), present the general discussion, giving an comprehensive overview of the performed work and elaborate on future application of probiotic bacteria in modulating immune responses in health and disease.

Abbreviations

Antigen presenting cells (APC)
Crohn's disease (CD)
C-type lectin receptors (CLR)
Dendritic cells (DC)
Dextran sulfate sodium induced (DSS)
Follicle-associated epithelium (FAE)
Gastro-intestinal tract (GI-tract)
Genome wide association studies (GWAS)
Inflammatory bowel disease (IBD)
Intestinal epithelial cells (IEC)
Lipopolysaccharide (LPS)
Laminapropria (LP)
Mesenteric lymph nodes (MLN)
Microfold cells (M-cells)
Muramyl dipeptide (MDP)
Nucleotide binding oligomerization domain receptors (NOD-like receptors)
Pathogens associated molecular pattern (PAMP)
Pattern recognition receptors (PRR)
Peyer's Patches (PP)
Toll-like receptors (TLR)
Trinitrobenzene sulfonic acid (TNBS)
T regulator (Treg)
Ulcerative colitis (UC)

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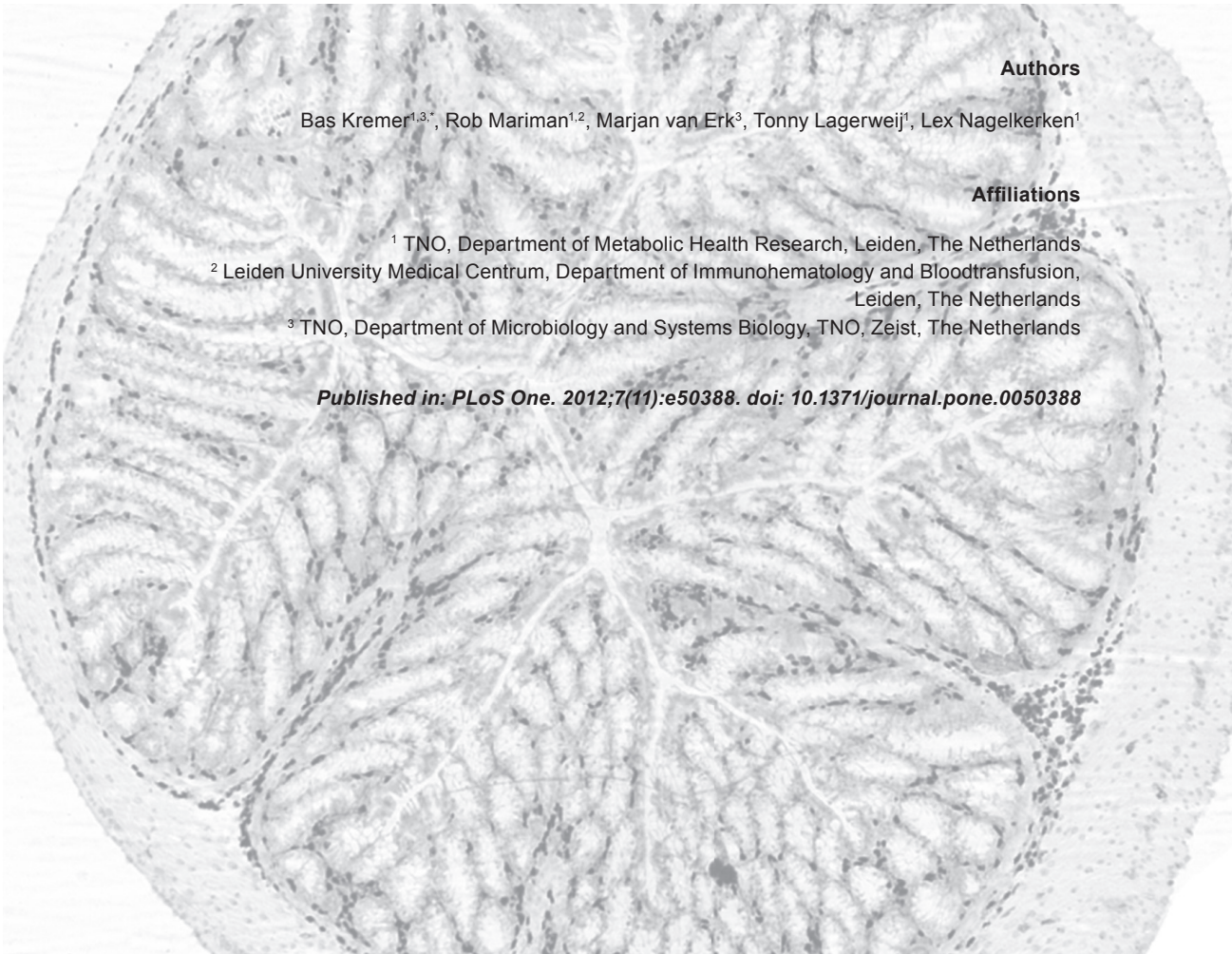
Chapter 2

Temporal colonic gene expression profiling in the recurrent colitis model identifies early and chronic inflammatory processes



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Abstract

The recurrent TNBS-colitis model in BALB/c mice has been proposed as a model of Inflammatory Bowel Disease with a shifting pattern of local cytokines with the expression of Th1 cytokines during the early phase, Th17 cytokines during the intermediate phase and Th2 cytokines during late fibrotic stages. In this study, we evaluated the development of pathology in time-in conjunction with genome-wide gene expression in the colons-, in response to three weekly intrarectal instillations of TNBS. During this time-frame mice develop colitis with extensive cellular infiltration of (sub)mucosa and mildly to moderately affected crypt architecture. These pathological processes were sensitive to local treatment with budesonide. Gene expression profiling confirmed an acute phase response after each intrarectal TNBS-challenge. In addition, a chronic inflammatory process developed over time particularly evident from a gradual increase in expression of mast cell related genes. The changes in pathological hallmarks were consistent with a temporal expression of mRNA encoding a selection of chemokines. In conclusion, the early stages of the recurrent TNBS-colitis model reflect several aspects of inflammatory bowel disease which are sensitive to immunomodulation.

Introduction

Inflammatory Bowel Diseases (IBD), with Ulcerative Colitis (UC) and Crohn's Disease (CD) as major entities, are chronic inflammatory disorders of the gastrointestinal tract, affecting an estimated 3.6 million people in Europe and the US [1]. IBD may be chronic or relapsing in nature, possibly due to a process of inflammation following an exaggerated immune response against enteric microorganisms. These processes affect intestinal function leading to diarrhea, cramping, and abdominal pain. Although considerable progress has been achieved with regard to the multifactorial nature of IBD [2], current therapies show limited efficacy [3].

Although there is a clear medical need to develop novel anti-inflammatory therapy for IBD, the evaluation of novel therapeutic strategies is hampered by the availability of suitable preclinical efficacy models. Most frequently, acute colitis models such as dextrane sodium sulphate (DSS) and the 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis are used [4,5].These models are characterized by severe acute inflammation of the colon representing histopathological aspects that reflect human IBD. Whereas the DSS-colitis model involves activation of the innate immune system in particular, the TNBS model was shown to depend on a local Th-1 biased response [6]. The acute chemically induced IBD models have been shown of great value for a better understanding of acute inflammatory disease processes in IBD. In addition, efficacies of a limited number of relevant treatments have been reported in these models [7-9].

As an alternative to chemically induced colitis, several models helped to gain valuable insight into the underlying pathology of IBD, such as the model based on transfer of $CD45RB^{Hi}$ $CD4^{+}$ T cell to immunodeficient recipients or the development of colitis in IL-10 deficient mice [10,11]. These models put emphasis on the role of the intestinal microbiota eliciting a chronic inflammatory response in the gut. However, because these models are performed in an immunodeficient environment with disturbed immunoregulatory circuits they are not always suitable for the evaluation of novel therapeutic strategies.

In recent years, several groups have demonstrated that repeated intrarectal administrations of low-dose TNBS to mice and rats result in chronic colitis [12,13]. This colitis model has primarily been used to study fibrotic processes as a consequence of chronic intestinal inflammation [14,15]. Recently, we have implemented a modified protocol of the chronic TNBS colitis model with reduced duration, in which treatment with probiotics was shown to prevent the onset of chronic colitis and associated clinical symptoms, suggesting sensitivity to immunomodulation [16] .

In this report, we characterize the processes underlying the development of pathology in this model by temporal gene expression profiling in the colon with the aim to further substantiate the value of this model for both mechanistic and pre-clinical efficacy studies. We show that

the recurrent TNBS-induced colitis model combines histopathological features with aspects of active inflammation and chronic processes of cellular infiltration, angiogenesis and tissue remodeling, which are all relevant to human IBD.

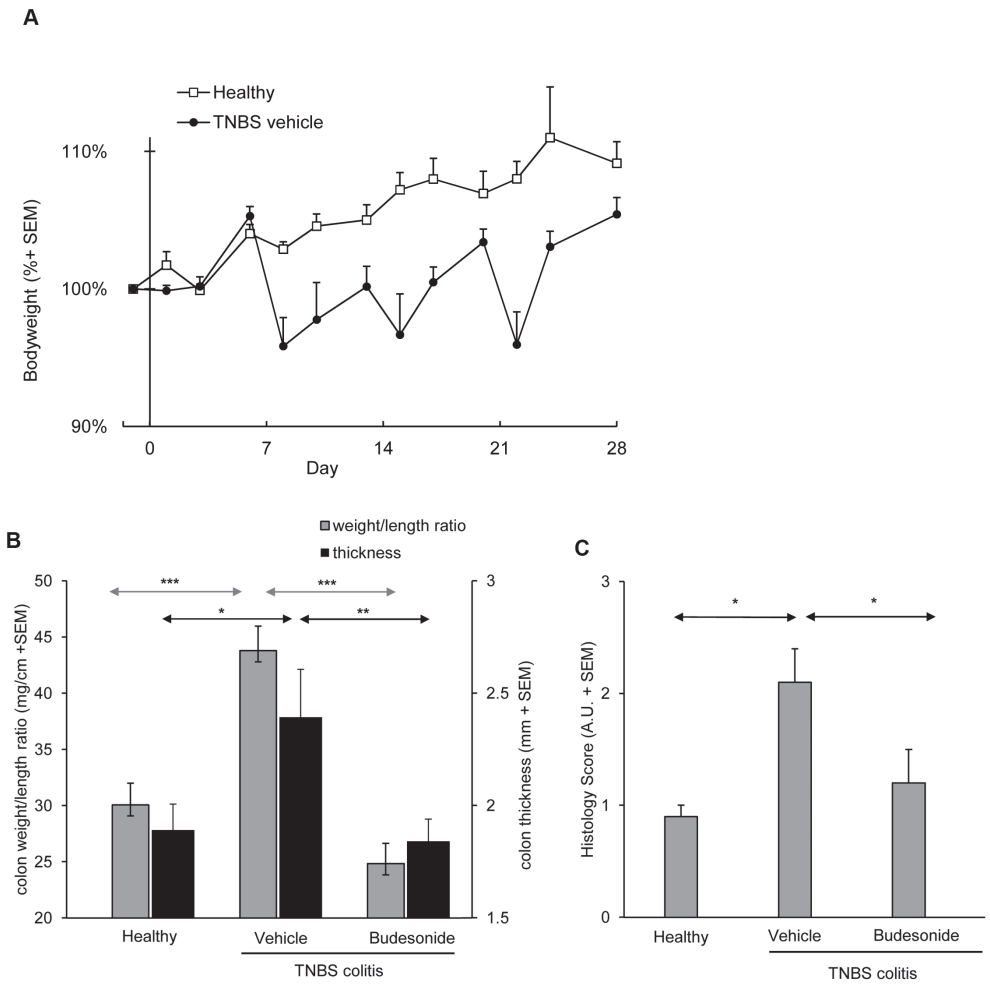

Figure 1. Disease characteristics of the recurrent TNBS model and efficacy of budesonide treatment.
The development of colitis induction is evident from periods of loss of body weight following each rectal instillation of TNBS (Figure 1A; AUC $p<0.005$). At end-point, inflamed colons show significantly increased weight/length ratios and colon thickness (Figure 1B); both aspects are suppressed by budesonide. TNBS colitis induction is associated with a significantly increased histological score (Figure 1C); this score is significantly lower in budesonide treated animals. Bars represent group mean values of 10-12 mice / group, error bars represent SEM. Mann-Whitney U-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 1. Relative circulating cytokine concentrations in TNBS-induced colitis[a]

	Group		
Cytokine	**Healthy (n=14)**	**Vehicle (n=19)**	**Budesonide (n=15)**
Eotaxin	100% ± 5%	102% ± 9%	89% ± 10%
G-CSF	100% ± 10%	114% ± 9%	83% ± 8%[c]
GM-CSF	100% ± 4%	108% ± 5%	98% ± 7%
IFNγ	100% ± 10%	137% ± 12%[b]	93% ± 14%[c]
IL-10	100% ± 25%	194% ± 37%	54% ± 16%
IL-12p40	100% ± 8%	87% ± 7%	28% ± 4%[c]
IL-12p70	100% ± 15%	117% ± 19%	89% ± 11%
IL-13	100% ± 9%	133% ± 12%	82% ± 10%[c]
IL-17	100% ± 13%	153% ± 17%[b]	94% ± 12%[c]
IL-1α	100% ± 19%	93% ± 12%	87% ± 17%
IL-1β	100% ± 8%	135% ± 12%[b]	82% ± 9%[c]
IL-2	100% ± 11%	118% ± 12%	84% ± 12%
IL-3	100% ± 13%	137% ± 14%	104% ± 13%
IL-4	100% ± 15%	111% ± 12%	85% ± 14%
IL-5	100% ± 6%	111% ± 12%	72% ± 8%[c]
IL-6	100% ± 15%	122% ± 9%	82% ± 9%[c]
IL-9	100% ± 9%	118% ± 8%	84% ± 8%[c]
KC	100% ± 7%	107% ± 7%	81% ± 4%[c]
MCP-1	100% ± 9%	131% ± 9%[b]	86% ± 16%[c]
MIP-1α	100% ± 8%	95% ± 7%	96% ± 10%
MIP-1β	100% ± 6%	121% ± 6%[b]	100% ± 10%
Rantes	100% ± 6%	91% ± 5%	80% ± 7%
TNFα	100% ± 10%	106% ± 9%	116% ± 20%

[a], In two independent studies, serum concentrations of 23 cytokines were determined by multiplex technology on day 28. In supportive table 2 concentrations are presented as pg/ml ± SEM. In each study, values were normalized and expressed as a percentage of the mean concentration of the corresponding healthy control mice. Relative concentrations and shown as % ±SEM.
[b], significantly ($p<0.05$) different relative serum concentration from that in healthy mice
[c], significantly ($p<0.05$) different relative serum concentration from that in vehicle treated mice.

Results

Recurrent TNBS-colitis: a model with low mortality sensitive to intrarectal budesonide

In this study, we evaluated early stages of the recurrent TNBS colitis model and confined ourselves to 4 weeks of follow-up after skin sensitization and three weekly intrarectal challenges with increasing dosages of TNBS, thereby combining two previously published protocols [5,15]. This adapted protocol consistently resulted in mild to moderate colitis, reflected by significant growth retardation($p <0.005$) (Figure 1A).Each rectal TNBS instillation

induced transient loss of body weight of 5 to 15%. Average mortality was less than 15 % and in particular associated with the first rectal challenge. At endpoint, colitis was evident from an increased colon weight/length ratio and increased thickening of the colon(Figure 1B). These macroscopic changes were associated with increased histology scores in the distal part of the colon (Figure 1C). Infiltration of mucosa and submucosa by $CD4^+$ and $CD8^+$ T cells, $CD11b^+$ cells (Figure 2A), confirmed as macrophages by F4/80 staining (Figure 2B) was associated with mild to moderate damage of the mucosal architecture.

All of these aspects were suppressed in mice treated by intrarectal administration of budesonide. Besides, budesonide treatment was associated with weight loss, typical for chronic exposure to corticosteroids in mice.

Multiplex analysis of 23 different cytokines and chemokines in serum collected at endpoint revealed significantly increased levels of IFN-γ, IL-1β, IL-17, MCP-1 and MIP-1β in mice with colitis, but not in mice treated with budesonide. Furthermore, budesonide suppressed IL-12p40 levels even compared to the serum concentrations in healthy control mice (Table 1).

Altogether, several inflammatory characteristics relevant to IBD are represented in this model and shown to be sensitive to corticosteroid treatment.

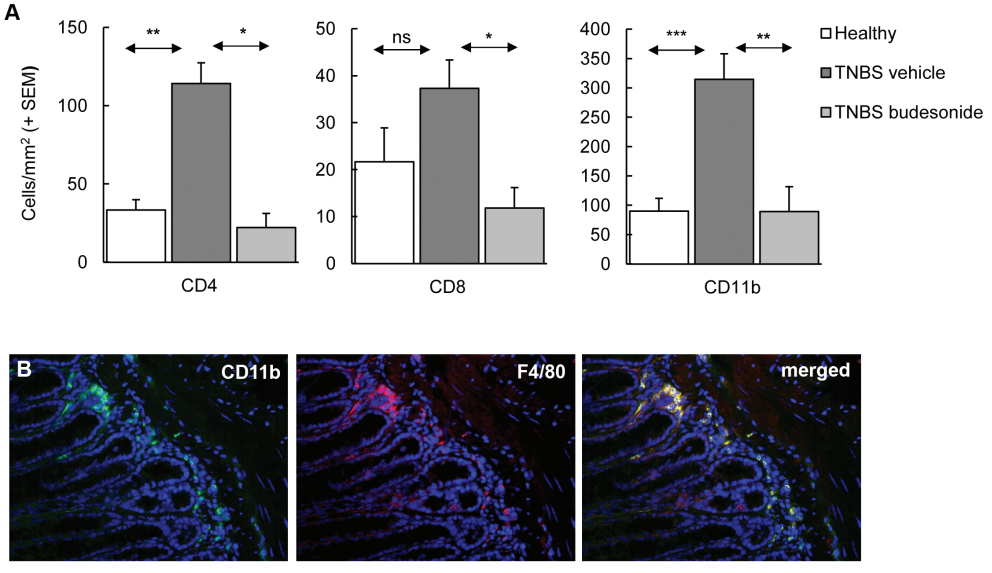

Figure 2. Colitis induction is associated with increased numbers of innate and adaptive immune cells.

The composition of the cellular infiltrate and the effect of budesonide treatment were analyzed by immunohistochemistry. **(A)** Enumeration of the cells showed significantly increased cell numbers per mm^2 tissue due to TNBS colitis induction. Budesonide treatment significantly reduced the number of these infiltrating cells. Bars represent group mean values of 10-12 mice / group, error bars represent SEM. Mann-Whitney U-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **(B)** Double staining of CD11b+ and F480+ cells using fluorescence microscopy confirmed co-expression of these two cell surface markers.

Alterations in gene expression during the development of recurrent TNBS colitis.
Because the adapted recurrent TNBS colitis model showed involvement of relevant pathological processes and sensitivity to treatment, we sought to gain more insight into processes involved in the early development of colitis in this model. Therefore, we evaluated gene expression in colons of mice sacrificed at different time-points, i.e. before the first and 2 or 7 days after each rectal TNBS instillation. Evaluation of colon macroscopy at each of these time-points showed that TNBS administration was associated with transient shortening of the colons, whereas colon weight increased gradually in time (Figure 3). Histological evaluation of the colons revealed marked areas of hemorrhages and complete destruction of crypt architecture two days after each TNBS instillation; this mucosal damage had partially recovered seven days after each instillation (data not shown).

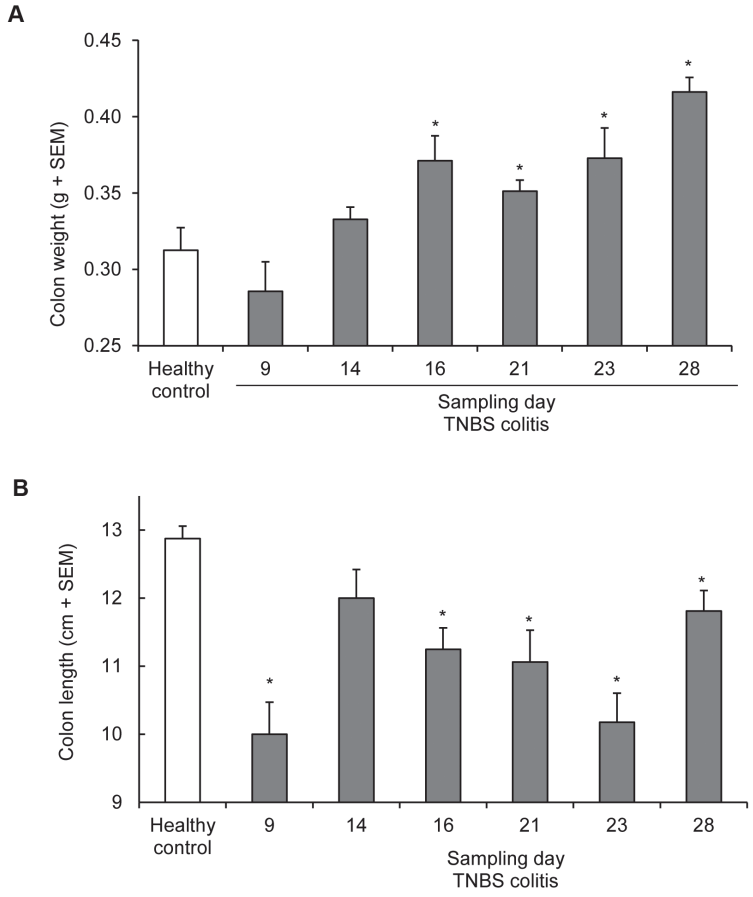

Figure 3. Disease characteristics of the recurrent TNBS model in time.
Results of the time-course study in the recurrent TNBS colitis model. At each time-point indicated in Figure 8, 8 animals were sacrificed and colon length **(A)** and weight **(B)** was determined. Colitis is associated with a gradual increase in colon weight whereas the colon length is severely affected by each TNBS administration followed by a gradual relaxation.

Genome wide expression profiling was performed on RNA isolated from distal colon tissue of five TNBS-treated mice at each selected time-point. Based on False Discovery Rate (FDR) correction, a total of 2074 genes of the 14285 transcripts that passed the filtering procedure,

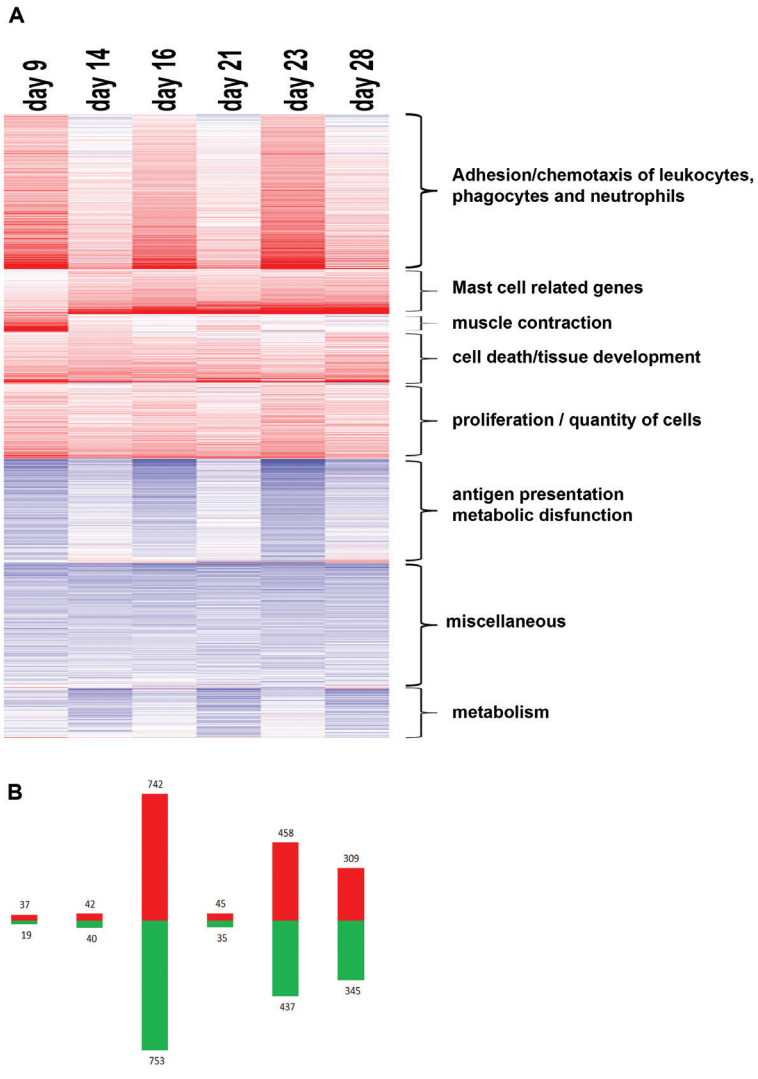

Figure 4. Transcriptome analysis in the recurrent TNBS-induced colitis model.
To determine temporal patterns of gene expression levels that were affected by the repeated TNBS instillations, microarray analysis was performed on RNA isolated from colon tissue of colitic mice at day 9, 14, 16, 21, 23, and 28 (n = 5 / group) **(A)** A heat-map of the genes with significantly modified gene expression revealed multiple clusters with different temporal expression patterns. **(B)** Bar graph indicates the number of up regulated (red) and down-regulated genes during colitis development at each time point. Genes were considered significant with a False discovery rate (FDR) p value <0.05. FDR was used to correct for multiple comparisons

Table 2. Master regulators in colitis development

Transcription factor	Central regulator of	Time-point					
		9	14	16	21	23	28
SP1	Lipid metabolism, inflammation		++	++		++	++
HNF4α	Endodermal development			++		++	
c-myc	cell proliferation	+	+	+		+	+
CREB1	Inflammation			+		+	+
p53	Apoptosis, cell cycle	+	+	+		+	+
NF-κB	Inflammation	+	+	+	+	+	
AP1	Inflammation			+		+	+
C/EBP-β	Lipid metabolism, inflammation			+			+
ESR1	Metabolism, inflammation					+	+

[a] Transcription factors were identified in biological networks generated using MetaCore™ software. Transcription factors represented in this table were identified in significant networks containing a minimal of 8 nodes.

[b] Level of significance of the transcription factor network is classified based on z-scores: +, z-score between 50 and 100; ++, z-score above 100. No score means that the networks for these transcription factors were not identified at the time-point or had a z-score below 50.

showed significantly different levels of mRNA at one or more time-points in comparison to mRNA from healthy mice. Differentially expressed genes that showed similar expression profiles are clustered in a heatmap (Figure 4A). The first TNBS challenge was associated with enhanced expression of a limited number of genes, whereas enhanced expression of a large number of genes occurred shortly after the second challenge on day 16 (Figure 4B). These patterns were largely normalized on day 21. Importantly, the third challenge resulted in extensive changes in gene expression on days 23 and 28. Forty-two % of the genes that showed increased expression on day 28 were also enhanced on day 23 and therefore not normalized. Furthermore, 27% of the genes differentially expressed on day 28, were not different from healthy on any of the earlier time-points, suggesting developing pathology upon multiple TNBS instillations.

To further elucidate the cellular processes associated with the induction of colitis by repeated TNBS instillations, we performed gene ontology analysis on the FDR filtered gene data set using MetaCore™ software. Enriched processes with more than 15 genes and p-values below 10^{-5} were considered to be significant (Supportive Table 1), which include general terms like “inflammation”, but also detailed underlying subcategories. Five major processes important for human pathogenesis of IBD were identified in this model, i.e. I) tissue morphogenesis, II) the response to wounding, III) immune/inflammatory responses, IV) cell adhesion and V) angiogenesis. Gene expression associated with immune/inflammatory responses was particularly evident 2 days after each TNBS challenge. Furthermore, several processes gradually developed in time, in particular processes associated with epithelial cell growth

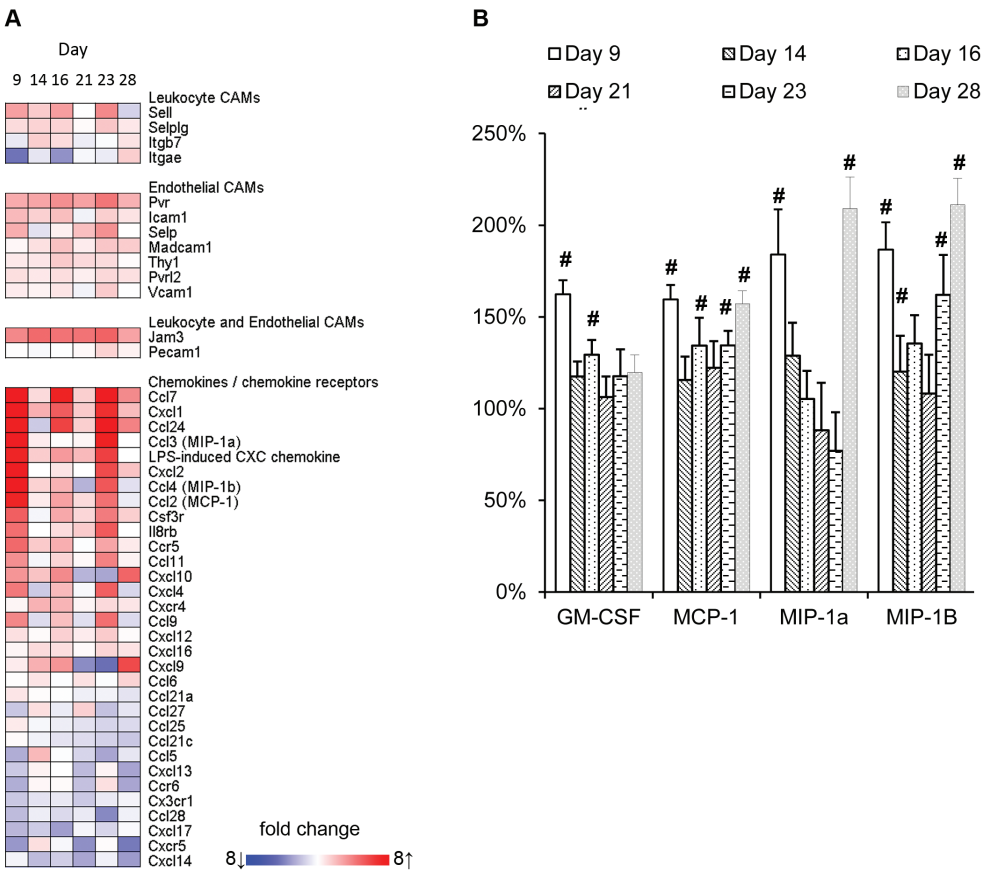

Figure 5. Effect of recurrent TNBS administrations on cell adhesion molecules and chemokines.
(A) Local mRNA expression of cell adhesion molecules and chemokines was determined within the time-course microarray experiment. The size of the effect of TNBS-induced colitis on gene expression in comparison to healthy colons is indicated in a heatmap. These differences are based on mean expression levels of 5 individual animals per time-point. **(B)** Circulating serum chemokines were analyzed by multiplex technology. Relative serum concentrations of concentrations of GM-CSF, MCP-1 (CCL2), MIP-1α (CCL3), and MIP-1β (CCL4) are graphically depicted; significant differences are indicated by #.

and tissue repair/wound healing (Supportive Table 1). Altogether, several processes found in human IBD are reflected in this recurrent TNBS model and these will be discussed more detailed in the next paragraphs. The key transcription factors involved in the processes underlying the development of colitis in this model were identified by MetaCore™ analysis. As shown in Table 2, Nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*), activator protein 1 (*AP-1*), CAMP responsive element binding protein 1 (*CREB1*), were central to the process at most time-points, in accordance with an inflammatory process.

Networks involving Hepatocyte-Nuclear Factor-4α (*hnf4α*) were only observed on day 16 and 23, consistent with its role in the acute phase response.

Chemokines, cytokines and cell-adhesion molecules in recurrent TNBS-colitis

Each of the repeated challenges with TNBS was followed by increased expression of genes encoding mediators of acute inflammation such TNF-α, IL-1β, CHI3L1, as well as calprotectin or calgranulin C (S100a8 -a9). These S100-proteins are relevant to the pathogenesis, disease activity, diagnosis, and therapeutic management of IBD [17].Apart from this acute response towards TNBS instillations, the adaptive arm of the immune system appeared to be of importance from day 23 onwards as shown by gene ontology analysis in Metacore™. This analysis also revealed a role for cell adhesion molecules and chemokines. The KEGG list of 69 cell adhesion molecules (CAMs), chemokines and chemokine receptors, that was previously applied to illustrate an important role of these molecules in mucosal biopsies of UC and CD patients [18], was used to evaluate their expression during the development of recurrent TNBS-colitis. A heat-map of the molecules on this list that showed significantly altered expression at one or more time points is presented in Figure 5A. Several endothelial and leukocyte CAMs that were increased in patients with colonic IBD were also enhanced in the mouse model, e.g. CD62L, ICAM1, MADCAM1, SELE, THY1, JAM3, and PECAM1. Moreover, several chemokines involved in the recruitment of leukocytes, i.e., CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL7 (MCP-3), CCL9 (MIP-1γ), CCL24 (eotaxin-2), CXCL-1 (KC), and CXCL-2 (MIP-2α) were induced as well. Not all transcripts related to immune responses were up-regulated. Several genes encoding chemokines, including CCL21, and 25, and CXCL12, 14, and 15, showed gradually decreased expression during the development of colitis, indicative of a changing chemotactic profile during the progression of colon inflammation. Multiplex analysis on serum samples revealed that concentrations of GM-CSF, MCP-1, MIP-1α, and MIP-1β were significantly increased at one or more time-points during the experiment, although they did not reflect colon tissue gene expression profiles for these chemokines (Figure 5B).

As reported previously, repeated challenges with TNBS are associated with a changing cytokine profile in colon, suggesting for a shift from Th1 to a Th17 profile, and eventually Th2 cells with fibrosis [15]. In line with this study, IFN-γ gene expression was increased in our study, with a maximum on day 16, whereas IL-4 gene expression remained unchanged until the third TNBS challenge, after which a significant increase was observed (Figure 6A). However, mRNA expression profiles of cytokines considered significant for IBD or colitis, such IL-12p40, IL-23p19, IL-12p35, IL-17 did not differ from those in healthy tissue (data not shown).

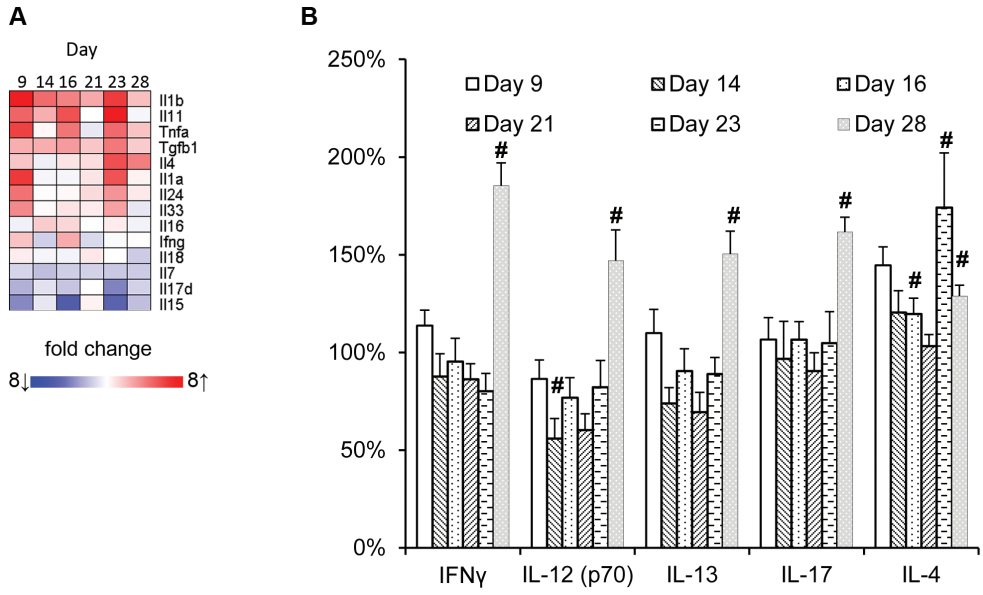

Figure 6. Effect of recurrent TNBS administrations on local and circulating cytokine profiles. **(A)** Local mRNA expression of cytokines was determined within the time-course microarray experiment. The size of the effect of TNBS-induced colitis on gene expression in comparison to healthy colons is indicated in a heatmap. These differences are based on mean expression levels of 5 individual animals per time-point. **(B)** Relative serum concentrations of Th1/Th2/Th17 hallmark cytokines at different time-points during the development of colitis. Concentrations of IFN-γ and IL-12p70, IL-4 and IL-13, and IL-17 are graphically depicted; significant differences are indicated by #.

To establish whether these IBD-related cytokines were reflected in the periphery, serum samples were subjected to cytokine analysis employing multiplex technology. A shown in Figure 6B, we did not obtain evidence for a biased cytokine profile at any time-point.

Involvement of mast cells and α-defensins in TNBS-colitis

A small subset of genes showed gradually increasing expression levels in time. This cluster mainly comprised mast cell specific genes (Figure7A), including mast cell protease 1, 4, and 6 (*mcpt1, 4, 6)*, mast cell chymase (*cma2,*), carboxypeptidase A3 (*cpa3*), tryptase α/β1 (*tpsab*), and high-affinity IgE Fc receptor (*fcer1a*).Gene expression was induced up to 233-fold (*mcpt1,* day 28). The highest expression levels of these genes were observed during the later stages of this model, i.e. on days 23 and 28. Immunohistochemical staining of FcεRI+ confirmed the presence of large numbers of mast cells in the lamina propria of affected colon tissue (data not shown). This was in line with increased numbers of mast cells stained by toluidine blue in affected colon tissue (Figure 7B).These results suggest a gradual increase of mast cells associated with the development of chronic colitis in this model.

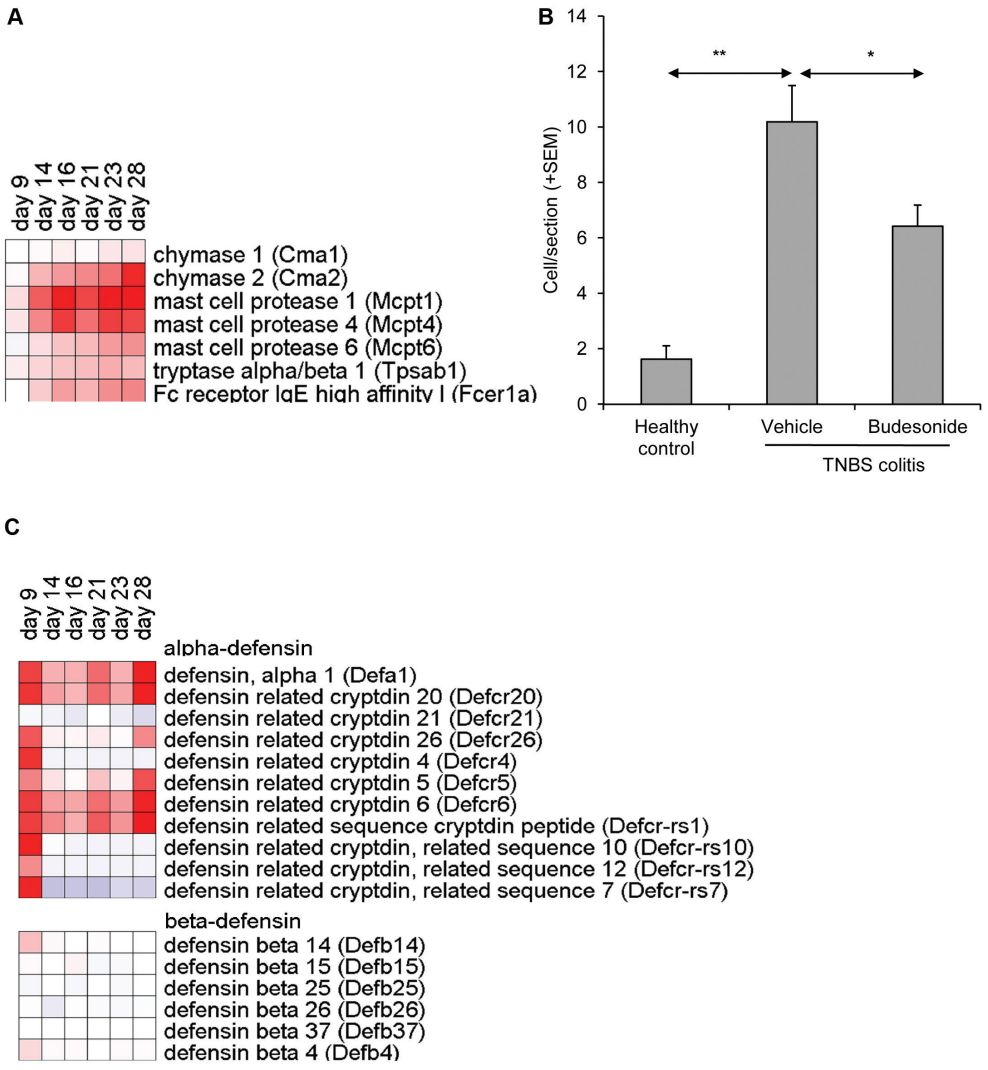

Figure 7. TNBS –induced colitis is associated with increased numbers of mast cells and α-defensins.
(A) mRNA expression of selected mast cell specific genes in affected colon tissue (n = 5 / group). Heatmap represents fold changes in TNBS colitis mice as compared to healthy control mice.
(B) Quantification of toluidine blue positive cells confirmed TNBS induced up-regulation of mast cells.
(C) mRNA expression of α- and β-defensin genes in affected colon tissue.

Another set of genes that showed strongly affected expression levels encoded for antimicrobial peptides. Expression of many members of the α-defensin family was transiently increased on day 9, immediately following the first intrarectal TNBS instillation (Figure 7C).A subset of these defensins, including α-defensin 1 (*defa1*) and four α-defensin-related cryptidins (*defcr5*, *6*, *20* and *21* and *defcr-rs1*) also showed increased expression on day 28. These genes were induced up to 132-fold (*defcr-rs1*, day 28).This modulation was restricted to α-defensins, as the level β-defensin gene expression remained unchanged.

Discussion

To gain insight into the pathogenesis of IBD, a variety of animal models reflecting different aspects of the disease are used [10,11,19]. Most IBD models result from exogenous manipulation; they can be categorized based on induction by chemicals, immune cell transfer, or gene targeting [19]. Models based on immune cell transfer, such as the $CD45RB^{Hi}$ T-cell transfer model, and models based on gene targeting, such as the IL-10 knockout mouse model, present excellent models to study mechanisms involved in the pathogenesis IBD [20]. Nonetheless, these models have the disadvantage of lacking a fully functional immune system. These limitations do not apply for models of colitis induced by DSS or TNBS. However, these models usually have the disadvantage of being mediated by damage and being relatively insensitive to anti-inflammatory drugs. In this respect the recurrent TNBS colitis model is more promising. This model has been proposed because the induced pathology reflects the chronic and relapsing immune activation processes underlying human IBD resulting in late onset of fibrosis [13,15]. In this report, we show results obtained in a modified version of this model, limiting the duration to first three weeks of colitis development. We show its sensitivity to immunosuppression by local corticosteroid treatment with budesonide which is a relevant compound in clinical treatment of patients with CD. Moreover, we recently showed that the model is sensitive to pretreatment with probiotic bacteria [16].

Our initial work to develop a model for colitis was largely inspired by the work of Neurath et al. who showed that TNBS-induced colitis is mediated by Th1 cells [21]. However, in our hands this model showed in SJL mice substantial mortality and variation, which was partly explained by the source of the animals and the type of chow diet (data not shown). Modifications to the protocol, as indicated by Wirtz *et al* [5], i.e. skin sensitization with TNBS prior to intrarectal challenges with TNBS, decreased the mortality rate in this model. Nevertheless, the limited therapeutic window of 2 days in the acute model prompted us to evaluate the characteristics of a recurrent TNBS-colitis model described by Fichtner-Feigl *et al* [15], allowing for prolonged treatment during a period of several weeks.

Our study further characterizes the processes underlying or associated with the early stage development of colitis in this model, i.e. a time frame of 3 weeks, by performing genome wide transcriptome analysis on colon tissue. This approach revealed that each intrarectal TNBS challenge induced a complex set of genes encoding molecules that are generally associated with an acute phase inflammatory response, such as TNFα, IL-6, calgranulins S100a8 and 9, and reg3γ.In addition, each intrarectal TNBS challenge was associated with an enhanced expression of genes encoding a set of chemokines; MCP-1 (CCL-2), MIP-1α (CCL3) and MIP-1β (CCL4), CCL7, CCL9, CCL11, CCL24,CXCL2 and CXCL4, showed a similar acute phase expression pattern, consistent with the influx of inflammatory cells. Moreover, serum concentrations of GM-CSF, MCP-1, MIP-1α, and MIP-1β were significantly increased in particular two days after each TNBS challenge. These data suggest that each TNBS challenge induces transient chemokine production in the colon, which likely drives the attraction of innate and adaptive immune cells to the site of TNBS-induced tissue damage. The involvement of these chemokines is relevant for human IBD since the majority is also elevated in tissues of UC and CD patients [22-24].

In addition, CXCL1 and CXCL2, which are chemokines with strong angiogenic effects, were strongly induced by the first intrarectal TNBS challenge and this was followed by the enrichment of a large cluster of genes encoding factors that are involved in angiogenesis, including FGF, endothelin-1, and VEGF-D. These data are suggestive for a link between the process of inflammation and angiogenesis in this model. Importantly, angiogenesis has been described as an important component in the pathogenesis and potential target for treatment of IBD [25].

Interestingly, the expression of a set of genes encoding chemokines involved in homeostatic processes, including CCL21, CCL25, CCL27 and CCL28, was gradually down-regulated in our TNBS colitis model. As these chemokines are involved in mucosal lymphoid structure development [26], our findings suggest that normal lymphatic tissue regeneration is affected in the model. This was substantiated by the observation that colitic tissue contained lower levels of transcripts associated with B cells (Cd19, Cxcr5, Tnfrsf13c, and Pou2af1)and intraepithelial lymphocytes [27] (IL-15, IL-7, andCD3γ).

Many genes that showed reduced expression upon TNBS colitis induction were related to metabolic processes. These main affected metabolic functions based on gene expression shifts comprise of changes in oxidative stress response processes, methylation, lipid biosynthesis, and small molecule metabolism. These observations reflect the strong connection between immune and metabolic response systems in the gut, as described Shulzhenko *et al* [28].

Mast cell influx is one of the key features of the model presented in this paper, as observed by microarray and histological examination. The transcriptomics data revealed progressively elevated mRNA levels of genes encoding mast cell specific products such as proteases 1,

4 and 6 (mcpt-1, -4, -6), carboxypeptidase 3 (cpa3), and high affinity Fc receptor for IgE (FcεRI). Mast cell infiltration was confirmed by increased numbers of toluidine blue positive mast cells as well as increases in FcεRI+ cells in the affected lamina propria. The attraction of mast cell progenitors from the blood depends on several factors, in particular local chemokine expression. These include, CCL2, CCXL4, CCL3, CCL24, and CCL11 [26,29]. These chemokines were up-regulated during acute inflammation phases of the model and may therefore contribute to the temporal migration/recruitment of these cells to the inflamed mucosa. Interestingly, protective effects of probiotics in this model were associated with reduced expression of these chemokines [16].Also in biopsies of CD and UC patients mucosal accumulation of mast cells has been observed [30-33];however, the causative relationship between the development of IBD and numbers or activation state of mast cells has not been established. As mast cells are known to produce and release a plethora of mediators involved in pro-inflammatory as well as regulatory and active tissue repair processes [32,34,35], we hypothesize that they may exert differential effects depending on the stage of the inflammation processes.

The induction of colitis was associated with an increased expression of a cluster α-defensins together with the expression of other antimicrobial peptides, such as angiogenin 4 (ang4), regenerating islet derived antimicrobial peptides RegIIIγ and RegIV (reg3g and reg4, respectively), and secretory phospholipase A2 type IIA (pla2a2a/pla2g2a). Although all of these anti-microbial peptides have been described as products of Paneth cells in the small intestine, we have not been able to demonstrate the origin of these transcripts in the colon. Staining with Lendrum'sphloxine-tartrazine or immunohistochemical staining for lysozyme P did not confirm the presence of Paneth cells in the affected colon tissue(data not shown). It is tempting to speculate that these genes are indicative of the presence of metaplastic Paneth cells, which have been reported in both colonic CD and UC biopsies [36-38], whereas they are not found in healthy colon tissue[39,40].In patients these cells showed increased expression of human α-defensins DEFA5 and DEFA6 in the colon [22,38,41,42]. Additional studies are needed to establish the origin of the antimicrobial peptide gene expression in affected colon tissue.

In conclusion, this recurrent TNBS-colitis model, adapted to comprise both transient acute inflammatory processes and gradually developing chronic inflammation, reflects several clinical and histopathological features of human IBD. The temporal gene expression profiles generated in this study revealed distinct processes involved in the onset and progression of disease in this model, and this knowledge may help to identify novel targets and therapeutic approaches for IBD.

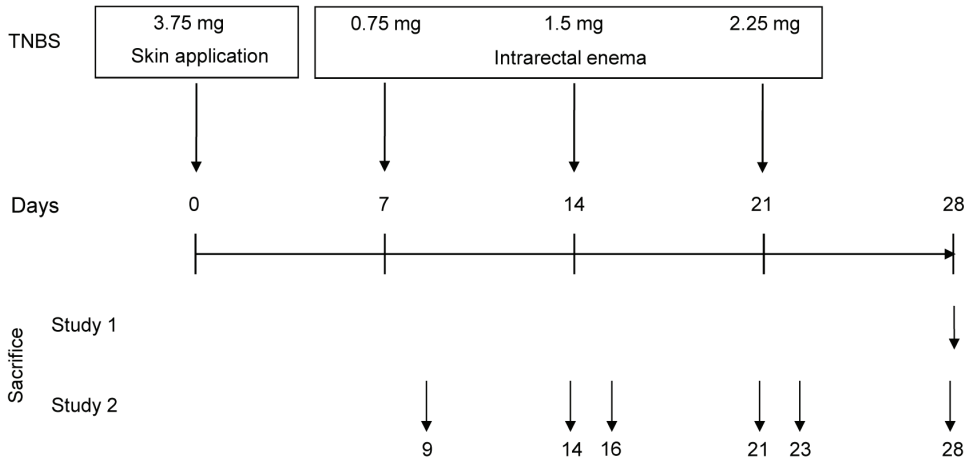

Figure 8. Schematic representation of the colitis induction protocol.
Induction of colitis is achieved by dorsal skin application of TNBS in ethanol on day 0, followed by rectal TNBS/ethanol instillations on day 7, 14, and 21. The necropsy time-points for study 1, i.e. TNBS disease induction and corticosteroid treatment, and study 2, i.e. the time-course experiment, are presented in the upper part of the schematic. Severity of colitis induction was assessed by measurement of colon weight, length and thickness and by histopathological evaluation of the distal part of the colon. At each necropsy, serum was collected for cytokine profiling and colon tissue was sampled for RNA isolation.

Materials and Methods

Mouse colitis model

All experiments were performed with female BALB/c mice (8wk old, 18-22 g) obtained from Janvier (St. Berthevin, France). Mice were conventionally housed under controlled temperature (22-24°C) and photoperiod (12hlight-dark cycle), and had free access to standard mouse chow (SSNIFF R/M-H, BioServices B.V., Uden, The Netherlands) and acidified tap water. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO), approval number DEC2982 and were in compliance with European Community specifications regarding the use of laboratory animals.

To induce colitis, mice were exposed to intracolonic administration of step-wise increasing doses of TNBS (Figure 8).Seven days prior to the first intracolonic TNBS administration, mice were sensitized by a single application of 3.75 mg of TNBS (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 48% (v/v) ethanol on the shaved dorsal skin. On days 7, 14 and 21, mice were lightly anaesthesized with isoflurane and subsequently 0.75, 1.5 and 2.25 mgTNBS in 40% (v/v) ethanol was administered per rectum via a 2.0 mm tube (Unomedical A/S,Birkerød, Denmark) connected with a 1.0 ml syringe, which was advanced into the rectum for

approximately 3 cm. Upon administration of 150 µl of TNBS solution, mice were held in a vertical position for 30seconds to allow for equal distribution.

Study design

In this report, the results of 2 experiments (Figure8) are discussed in detail. In study 1, induction of colitis was performed as described above and at day 28 all animals were sacrificed for assessment of all relevant parameters. In this study, local budesonide (3 mg/kg) treatment was included as a positive treatment control. Budesonide was administered 3× per week starting one day prior to the first rectal TNBS administration, i.e. day 6. Budesonide (Sigma-Aldrich, Zwijndrecht, The Netherlands) was prepared in PBS containing 2.5% (v/v) Tween-80 and 2.5% (v/v) ethanol. In study 2, we performed a time-course study to evaluate the development of colitis by macroscopy, cytokine profiling and gene expression profiling. In this study, one group of animals (n=8) was included for each time-point in the study. The selected time-points 2 and 7 days after each TNBS administration, 8 mice were sacrificed, as indicated in Figure 8.

Colon macroscopy

Severity of colitis was assessed by macroscopical analysis directly upon sacrifice. After colon length was measured, feces were removed by gentle pressure with forceps. Subsequently, colon weight was measured. Finally, colon thickness was measured with a digital caliper at 1, 2, and 3 cm from the anus.

Histological assessment of colitis

Following measurement of macroscopy parameters, colons were dissected and fragments were fixed in 4% buffered formalin and embedded in paraffin. Sections of 5 µm were stained by hematoxylin-eosin-saffron. Inflammation was scored in a blinded manner for 3 non-sequential sections per colon fragment at a 400× magnification, according to a semi quantitative (0-4) scoring system, based on number of infiltrating cells and mucosal damage. Mast cells were evaluated after staining with toluidine blue[43]at a 400× magnification.

Immunohistochemical analysis of cellular infiltrates

Dissected colon fragments were immediately immersed in Tissue TekOCT compound (Sakura Finetel, Torrance, CA, USA) and cryopreserved in liquid nitrogen. Immunohistochemistry was performed on 6 µm sections with primary antibodies specific for CD4 (L3T4.), CD8 (53-6.7), CD11b (M1/70), and F4/80 (CI-A3-1all obtained from BD Biosciences(San Diego, CA, USA). After incubation, biotinylated antibodies were detected by incubation with streptavidin-HRP (Vector Laboratories, Burlingame, CA, USA) using 3-amino-9-ethylcarbozole (Sigma) as a substrate. Immunopositive cells were counted at a 400× magnification and normalized

against the colon tissue surface area. For immunofluorescence, primary antibodies and according Texas red and fluorescein labeled secondary antibodies were applied. Slides were covered with Vectashield (H1000) mounting medium with DAPI Vector).

Cytokine and chemokine quantification in serum

Cytokine concentrations in serum obtained at each sacrifice were determined by Multiplex analyses. For this purpose, serum was diluted and assayed with the Bio-Plex Pro 23-Plex Panel, for IL-1α, -1β, -2, -3, -4, -5,-6, -9, -10, -12(p40), -12(p70), -13, -17, IFN-γ, TNF-α, RANTES, MIP-1α and -1β, MCP-1, KC, G-CSF, GM-CSF, and eotaxin protein (Bio-Rad Laboratories, Hercules, CA, USA). The beads were read on a LiquiChip 200, (Qiagen, Hombrechtikon, Switzerland), and data were analyzed by the five parameter curve fitting in Luminex100 IS Software. Cytokine concentrations were corrected for the dilution factor and presented in pg/ml or as relative concentrations to the average concentration in healthy mice.

Transcriptome analysis

Total RNA from frozen intestinal tissue was isolated using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. RNA was treated with DNAse and purified using Nucleospin RNAII Total RNA Isolation kit (Macherey-Nagel, Düren, Germany), according to manufacturer's protocol.

The quality control of RNA samples, RNA labeling and hybridization were performed at ServiceXS (Leiden, The Netherlands). RNA concentration was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A). The RNA quality and integrity was determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). The RNA integrity numbers (RIN) of all RNA samples had values above 7.3. BiotinylatedcRNA was prepared using the IlluminaTotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's specifications starting with 500 ng total RNA. Per sample, 750ng of cRNA was used to hybridize to the Sentrix MouseRef-8 BeadChips (Illumina, Inc., San Diego, CA, U.S.A.). Each BeadChip contains eight arrays and each of the arrays harbors 25697 probes. Hybridization and washing were performed according to the Illumina standard assay procedure. Scanning was performed on the IlluminaiScanner (Illumina, Inc., San Diego, CA, U.S.A.). Image analysis and extraction of raw and background subtracted expression data were performed with IlluminaBeadstudio v3 Gene Expression software using default settings. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[44] and are accessible through GEO Series accession number GSE35609(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35609).GeneSpring GX 11.0 was used for quantile normalization of the probe-level, background subtracted expression values. After the normalization, unexpressed probes were removed from the further analyses. All

expression values below 5 (2.322 on log2 scale) were floored to 5. Differentially expressed probes were identified using the limma package of the R/Bioconductor project, applying linear models and moderated t-statistics that implement empirical Bayes regularization of standard errors[45]. The statistical analyses were performed through The Remote Analysis Computation for gene Expression data (RACE) suite athttp://race.unil.ch [46]. False Discovery Rate (FDR) corrected p-values of 0.05 was used as threshold for significance of the differential expression. Fold changes relative to healthy control mice are presented. GEO Processes were identified using Metacore Software V6.11.

RT-qPCR for a set of genes (CCL2, CCL11, CCL24, cldn-1, cldn-4, cldn-11) showed a high correlation with data obtained by microarrays, and thereby confirmed the relative gene expression presented in the figures (data not shown).

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Chapter 3

Gene expression profiling identifies mechanisms of protection to recurrent TNBS-colitis mediated by probiotics

Authors

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Abstract

Background

Host-microbiota interactions in the intestinal mucosa play a major role in intestinal immune homeostasis and control the threshold of local inflammation. The aim of this study was to evaluate the efficacy of probiotics in the recurrent trinitrobenzenesulfonate (TNBS) induced colitis model, and gain more insight into protective mechanisms.

Method

Moderate chronic inflammation of the colon was induced in BALB/c mice by repetitive intrarectal challenges with TNBS. Administration of probiotics started two weeks before colitis induction, and was continued throughout colitis development.

Results

Long-term administration of *Lactobacillus plantarumNCIMB8826* or the probiotic mixture VSL#3 reduced intestinal inflammation induced by TNBS, evident from improved colon morphology and less influx of innate ($CD11b^+$) and adaptive ($CD4^+$/$CD8^+$) immune cells in the intestinal mucosa and decreased pro-inflammatory serum cytokines (IFN-γ, IL-17, IL-1β, MCP-1) in probiotic-treated mice. Genome wide expression analysis of colonic tissues using microarrays revealed differences in expression of genes related to inflammation and immune processes between untreated and probiotic treated mice. Principal Component Analysis revealed that probiotic treatment resulted in a shift of gene expression profiles towards those of healthy controls. Effects of probiotics on colonic gene expression were most profound during active inflammation, in particular on gene clusters related to mast cells and anti-microbial peptides. The results were substantiated by suppression of chemokine gene expression.

Conclusions

Our data are in favor of a model in which probiotics down-regulate expression of chemokines in the colon, thereby decreasing influx of inflammatory cells and rendering mice resistant to the induction of colitis.

Introduction

Inflammatory bowel disease (IBD) refers to chronic, spontaneously relapsing inflammation of the gastrointestinal tract, including Crohn's disease (CD) and Ulcerative colitis (UC) as two major entities. The exact etiology and pathogenesis of IBD is still unclear, but it includes genetic, immunological, and environmental factors (1;2). Standard therapies comprise salicylates, corticosteroids, immunomodulating and immunosuppressive drugs (3). However, these strategies are not effective in many patients and put the patients at risk to develop opportunistic infections or treatment related cancers. Alternative strategies such as altering the composition of intestinal microbiota with selected prebiotics, probiotics and/or synbiotics have been shown effective in inducing sustained remission of UC patients (4).

Interactions between host and microbiota play a key role in the susceptibility for experimental colitis, as germ-free mice do not develop colitis (5). The intestine is home to a complex population of trillions of commensal bacteria that participate in the digestive process and interact with the mucosal immune system of the host (6). The interaction of commensal bacteria with epithelial and immune cells in the intestine may cause both pro-inflammatory and regulatory immune responses(7). Therefore, altering the composition of the intestinal microbiota with probiotics has been suggested as an alternative approach in either maintaining or establishing intestinal homeostasis in IBD patients.

Probiotics are defined as viable, non-pathogenic micro-organisms that if ingested in sufficient numbers have a beneficial effect on the host. Different mechanisms have been suggested to explain this beneficial effect, such as colonization resistance, enhancing immune function by interacting with innate immune cells in the mucosa, and regulation of the intestinal barrier integrity (8). Indeed, clinical efficacy of probiotics has been proven in several studies in IBD patients and animal models of IBD.

In this study, we examined the effect of two probiotics, *L. plantarum* NCIMB8826 and VSL#3,on intestinal inflammation in the recurrent TNBS colitis model. VSL#3 was selected as a clinically validated product to identify pathways leading to a decreased inflammatory state. In this respect, we had a particular interest in the modulation of Toll-like receptors (TLR)and pathways involved in the recruitment of inflammatory cells. We are the first to perform genome wide screening of colonic tissue in this recurrent model of colitis in order to identify processes and pathways that are modulated by probiotics. This screening revealed that effects of VSL#3 treatment were most abundant during active inflammation, especially on gene clusters related to mast cells and anti-microbial peptides, and these effects were reflected by decreased influx of inflammatory cells.

Materials & methods

Mice

Eight-week old female BALB/c mice (18-21 gr) were obtained from Janvier (St. Berthevin, France) and maintained in the animal facility of TNO under specific pathogen free conditions with a 12h light/dark cycle. Mice had free access to standard mouse chow (SSNIFF R/M-H, Bioservices B.V., Uden, The Netherlands) and water. Animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Probiotic strains

VSL#3 (Ferring Pharmaceuticals, Berkshire, UK) was purchased as a commercially available probiotic mixture containing freeze-dried bacteria (*Bifidobacterium longum, B. breve,B. infantis, Lactobacillus acidophilus, L. plantarum, L. casei,L. bulgaricus, and Streptococcus thermophilus).*

*L. plantarum*NCIMB8826wasgrown at 37 ^{o}C in Mann-Rogusa Sharpe broth (ScharlauChemie, Barcelona, Spain) until mid-exponential growth phase (OD_{600nm} = 1). Bacteria were washed twice in phosphate buffered saline (PBS) at pH 7.4, suspended at 2×10^{9} cells/ml in PBS containing 25% glycerol, and stored at -80 ^{o}C until use. Before use, the bacterial suspensions were washed once with PBS. Mice were treated three times a week by oral administration of3 x10^{8} CFU *L. plantarum* or VSL#3 suspended in 200 µl PBS, or 200 µl vehicle (PBS) only, starting 14 days before the first intrarectal challenge with TNBS .

Induction of colitis

Colitis induction was performed as described previously(9), with slight modifications. Briefly, mice were sensitized by application of 3.75 mg of TNBS (Sigma-Aldrich) in 48% (v/v) ethanol to the shaved dorsal skin on day 0. Experimental colitis was induced by administration of increasing doses (0.75, 1.0 and 2.5 mg / mouse) of TNBS in 40% ethanol on days 7, 14 and 21, respectively. Mice were anesthetized with 3 % isoflurane and then administered TNBS in ethanol via a sterile 2.0 mm catheter (Unomedical A/S, Birkerød, Denmark)inserted 35 mm, intrarectally. Mice were kept in a head-down position for an additional 30seconds to ensure distribution of TNBS in the colon. On day 28, i.e. 7 days after the third TNBS challenge mice were sacrificed and colons were removed. After the removal of feces, colons were subjected to macroscopic evaluation before further processing for RNA isolation and histology.

Analysis of serum cytokines

Serum cytokine and chemokine protein levels were quantified using a Mouse Cytokine 23-PlexPanel (Bio-Rad,Hercules, CA, USA) according to manufacturer's protocol. The assay is based on multiplex technologyand simultaneously measures IL-1α, -1β, -2, -3, -4,

-5,-6, -9, -10, -12(p40), -12(p70), -13, -17, IFN-γ, TNF-α, RANTES, MIP-1α and -1β, MCP-1, KC, G-CSF, GM-CSF, and eotaxin protein. The beads were read on a LiquiChip 200, (Qiagen, Hombrechtikon, Switzerland), and data were analyzed by the five parameter curve fitting in Luminex100 ISSoftware.

Transcriptome analysis

Five mice per experimental group were selected for transcriptome analysis. The selection of animals was performed one day before sacrife and based on weight loss only. The average weight loss of the mice selected for transcriptome analysis was representative for all mice in each of the experimental groups. Total RNA was isolated from frozen colon tissue using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. RNA was treated with DNAse and purified using a nucleospin RNAII Total RNA Isolation kit (Macherey-Nagel, Düren,Germany). Concentrations and purity of RNA samples were determined with a NanoDrop ND-1000 spectrophotometer (Isogen, The Netherlands). RNA integrity was checked employing an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips according to the manufacturer's instructions. RNA integrity numbers (RIN) were above 8.

BiotinylatedcRNA was prepared using the IlluminaTotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, USA) according to the manufacturer's specifications starting with 500 ng total RNA. Per sample, 750ng of cRNA was used to hybridize to the Sentrix MouseRef-8 BeadChips (Illumina, Inc., San Diego, CA, USA). Each BeadChip contains eight arrays and each of the arrays harbors 25697 probes. Hybridization and washing were performed according to the Illumina standard assay procedure. Scanning was performed on the IlluminaiScanner (Illumina, Inc., San Diego, CA, USA). Image analysis and extraction of raw and background subtracted expression data were performed with IlluminaBeadstudio v3 Gene Expression software using default settings.

GeneSpring GX 11.0 was used for quantile normalization of the probe-level, background subtracted expression values. After normalization, unexpressed probes were removed from the subsequent analyses. Probes were considered to be expressed, if the probe was present (detection p-value >=0.99) in at least 3 out of 20 samples in the dataset.

After this filtering procedure, 13551 probes remained in the analysis. All expression values below 5 (2.322 on log2 scale) were floored to 5. Differentially expressed probes were identified using the limma package of the R/Bioconductor project, applying linear models and moderated t-statistics that implement empirical Bayes regularization of standard errors(10). The statistical analyses were performed through The Remote Analysis Computation for gene Expression data (RACE) suite at http://race.unil.ch(11). p-values below 0.05 were used as a threshold for significance of the differential expression. Pathway analysis software

Metacore(V6.2), a highly curated Web-based application for identification of gene ontology processes in input genesets, was used to identify biological processes (GeneGo Inc., St. Joseph, MI, USA).

RT-qPCR

Three genes encoding chemokines, which were upregulated during colitis, were selected for validation with RT-qPCR. 500 ng of RNA was used for single-stranded cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad,CA, USA) according the manufacturer's protocol. RT-qPCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR system. Primer sequences used were as follow; CCL2 5'-TAGGCTGGAGATCTACAAGAGG-3' (S) and 5'- AGTGCTTGAGGTGGTTGTGG-3' (AS), CCL11 5'-GGCTGACCTCAAACTCACAGAAA-3' (S) and 5'-ACATTCTGGCTTGGCATGGT-3' (AS), CCL24 5'- GCAGCATCTGTCCCAAGG-3' (S) and 5'- GCAGCTTGGGGTCAGTACA -3' (AS). Each reaction contained 25ngcDNA, 0.3μM sense and antisense primers, and Sybr Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) under the following thermal conditions: 50°C for 1 minute, 95°C for 10 minutes, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by a dissociation stage. Relative mRNA expression was normalized to β-actin and was expressed using the $\Delta\Delta C_t$ method.

Immunohistochemisty

Colon fragments adjacent to those used for RNA isolation were fixed in 4% buffered formaldehyde and embedded in paraffin, or embedded in Tissue-Tek and snap-frozen in liquid nitrogen.

From paraffin-embedded tissue,5 μm sections were stained with either toluidin blue and counterstained by hematoxylin-eosin, in order to assess numbers of mast cells or with hematoxylin-eosin-safran for microscopical evaluation.

Six μm cryo-sections from frozen colon fragments were fixed in ice-cold acetone for 10 minutes and air-dried. Sections were blocked for endogenous peroxidase (Peroxidase Blocking Agent, Dakocytomation, Glostrup, Denmark), avidin/biotin (SP-2001, Vector Laboratories, Peterborough, UK), and 5% BSA for non-specific antibody binding. Slides were incubated overnight at 4 °C with the following rat anti-mouse monoclonal antibodies (BD Biosciences, San Diego, CA, USA) in appropriate dilutions: CD4 (clone H129.19) and CD8-bio (clone 53-6.7), IgG2a isotype (clone R35-95); CD11b-bio (clone M1/70), FcRεI-bio, (clone MAR-1), IgG2b-bio isotype (clone A95-1). Bound primary antibody was detected via goat anti-rat-biotin and/or peroxidase-labeled streptavidin (SA-5004, Vector Laboratories). Slides were developed with Novared substrate (SK-4800, Vector Laboratories) and counterstained with haematoxylin. Three non-serial colon sections per mice were used for quantification, by counting immunopositive cells at a 400x magnification, which were normalized against the mucosal area.

Results

Treatment with probiotics reduce primary outcome parameters of recurrent TNBS-colitis

Mice were subjected to oral treatment with probiotics during the development of colitis or treatment with PBS (p.o.), starting 2 weeks before the first rectal TNBS challenge. During the experimental period, healthy control mice which were left untreated showed gain of bodyweight as compared to baseline (109.0±3.7%). In contrast, mice subjected to colitis induction showed a transient weight loss for 3 days immediately following each TNBS instillation (Figure 1A).At endpoint (day 28) mice subjected to colitis induction showed less gain in bodyweight (104±4.0%; $p<0.01$) as compared to the healthy controls. Although also the mice treated with *L. plantarum* or VSL#3 showed transient weight loss after each TNBS challenge, they showed less weight loss over the entire follow-up period compared to vehicle-treated mice challenged with TNBS (Figure 1A; repeated measurements ANOVA, $p<0.001$ and $p<0.01$ for *L. plantarum* and VSL#3, respectively).

Seven days after the third TNBS challenge (i.e. on day 28),mice were sacrificed and colons were evaluated macroscopally. Colons of vehicle-treated mice showed an increased weight/length ratio compared to healthy mice (Figure 1B; $p<0.001$). Colitis was also evident from thickening of the rectal part of the colon ($p<0.01$; data not shown).Although hemorrhages (as shown by histological evaluation) and the absence of solid stool are a hallmark during the acute phase following each rectal TNBS instillation, these features were less pronounced on day 28 and are therefore not included in the evaluation of treatment effects. Adhesions of the colon were only sporadically observed.

VSL#3 treated mice showed a less pronounced increase in the colon weight/length ratio as compared to vehicle-treated mice(Figure 1B; $p <0.05$) without having a significant effect on the colon thickness (data not shown). *L. plantarum* did not show effects on the macroscopic score. In a separate time-course experiment, VSL#3 was shown to be most effective after the second and third challenge (Figure 1D).

Histopathological evaluation of the rectal part of the colons was performed to establish a composite score as described before (12),based on mucosal architecture, cellular infiltration, muscle thickening, goblet cell depletion and crypt abscess formation. On day 28, in particular cellular infiltration and loss of mucosal architecture contributed to the score in mice with colitis compared to healthy controls (Figure 1C). Both VSL#3 and *L. plantarum* showed a modest inhibitory effect on this semi-quantitative score. As will be discussed below, significant inhibitory effects of probiotics were demonstrated after quantification of discrete subsets of cells.

FIGURE 1A

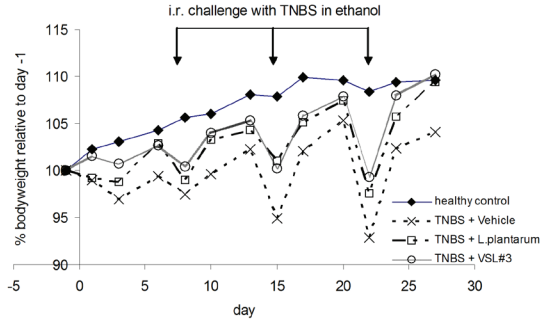

FIGURE 1B

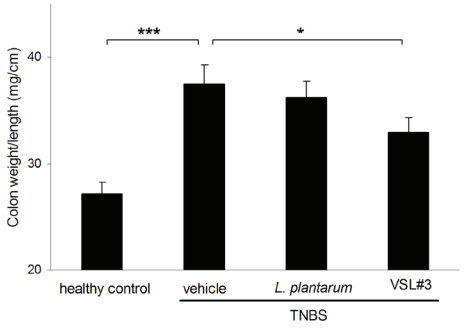

FIGURE 1C

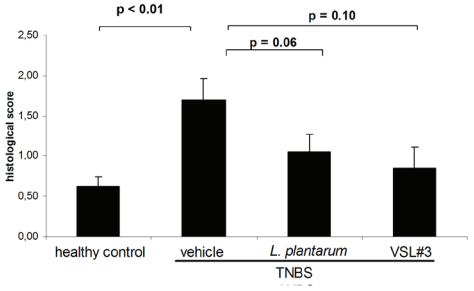

FIGURE 1D

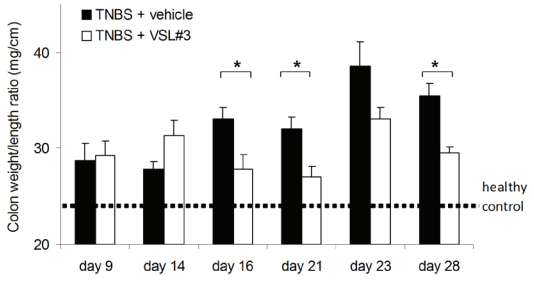

Figure 1.
(A) Body weight development in the recurrent TNBS colitis model. Body weights were normalized against the body weight on day -1. Colon weight/length **(B)** is a parameter reflecting changes in colon morphology (n = 10-12 mice / group). **(D)** Histological score of the colon. **(D)** Development of colon weight/length ratio in time (n = 8 mice per group). Panel A, B and D are representative for 2 independent experiments. Mann-Whitney U-test; * p<0.05, *** p<0.001.

Table 1. Effect of *L. plantarum* and VSL#3 treatment on TNBS induced up-regulation of α-defensins.

	TNBS VSL#3 vs TNBS vehicle		TNBS *L. plantarum* vs TNBS vehicle	
α-defensin	**Fold change**	**p**	**Fold change**	**p**
defensin related cryptdin 20 (Defcr20))	-9.7	0.006	-1.9	0.38
defensin related cryptdin 26 (Defcr26)	-5.0	0.009	-1.8	0.48
defensin related cryptdin 4 (Defcr4)	-5.4	0.026	-1.6	0.62
defensin related cryptdin 5 (Defcr5)	-4.8	0.031	-1.8	0.44
defensin related cryptdin 6 (Defcr6)	-11.2	0.002	-2.7	0.21
defensin related cryptdin, related sequence 10 (Defcr-rs10)	-4.8	0.069	-1.4	0.77
defensin related cryptdin, related sequence 7 (Defcr-rs7)	-5.0	0.031	-1.3	0.74
defensin related sequence cryptdin peptide (Defcr-rs1)	-13.8	0.004	-2.4	0.28
defensin, alpha 1 (Defa1)	-12.5	0.006	-2.3	0.34

Fold change is defined as the ratio of the signal of mice treated with TNBS and probiotic to that of mice treated with TNBS + vehicle.

Table 2. Probiotic treatment preventsinductionof various cytokines and chemokinesassociated with TNBS-colitis.

	Concentration of cytokine/chemokine in serum (mean pg/ml ±SD)			
	Healthy	**TNBS colitis**		
	vehicle	**Vehicle[1]**	***L. plantarum*[2]**	**VSL#3[2]**
G-CSF	53±42	45±19	42±20	26±10*
GM-CSF	185±42	214±43†	182±31†	169±29*
IFN-γ	530±240	763±184 †	643±242	463±170**
IL-10	63±51	196±101 *	126±49 †	121±73
IL-17	77±55	149±68 *	92±54 *	64±30 **
IL-1α	237±91	273±60	219±69 †	179±81*
IL-1β	1004±379	1499±547 †	1078±521 †	887±271 *
IL-3	11±5	19±16	10±3 *	15±4.3 *
IL-9	193±43	267±185	187±73	152±66 *
MCP-1	560±243	878±291 *	712±254	542±185 *
MIP-1α	1593±434	1929±399	1583±346	1348±268 **

Serum of individual mice, collected at endpoint on day 28, was assayed by multiplex analysis as described in material and methods.Results represent mean + SD of 6 to 10 mice per group.

[1] Mann-Whitney U-test as compared to healthy mice

[2] Mann-Whitney U-test as compared to vehicle-treated mice with colitis† p<0.10, * p<0.05, ** p<0.01

Genome wide gene expression analysis

To gain insight into colitis-associated processes modulated by *L. plantarum* and VSL#3, RNA from colonic tissue was isolated 7 days after the last TNBS challenge, and subjected to genome wide screening. Microarray analysis identified 831 probes that were differentially expressed between healthy mice and mice with colitis. Post-genomic validation of microarray data with RT-qPCR for 6 genes showed a strong correlation between both techniques (data not shown). The complete list of differentially expressed genes is provided in Supporting Table 1. The effect of probiotic treatment on TNBS colitis was visualized by principal component analysis (PCA) on the set of 831 probes that differed between healthy mice and mice with

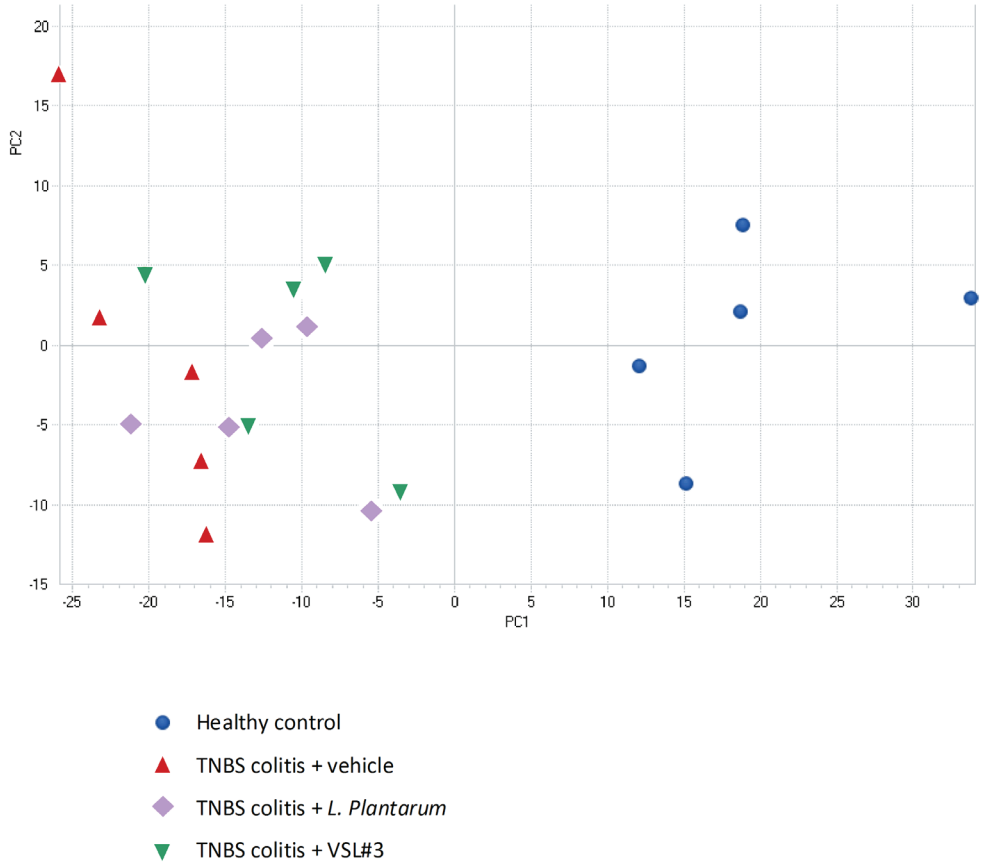

Figure 2.
Two-dimensional visualization of principal component analysis (PCA) constructed with the set of 831 differentially expressed genes in the colon upon colitis induction, and position of colitis mice treated with L. plantarum or VSL#3 in this space. Each dot represents the expression profile of an individual mouse.

colitis (Figure 2). PCA analysis allows grouping of individual mice with overall similar gene expression profiles. Although all these 831 genes contributed to the differentiation between healthy control mice and colitis animals, dominant clusters of genes encoded for mast cell enzymes, defensin related peptides, as well as other immune related transcripts. This plot showed that long-term probiotic administration to mice subjected to colitis induction exhibited a profile in between healthy and diseased mice, although still closer to diseased mice. To gain insight into disease-related processes that were modulated by probiotics, we next focused on genes and gene clusters affected by probiotic intervention during colitis. Gene ontology (GO) classification using Metacore pathway analysis was applied on genes that were normalized in probiotic treated mice. Cellular processes that were normalized by VSL#3 treatment were mainly related to DNA replication and regulation of immune functioning. Each intrarectal TNBS challenge was associated with up-regulation of gene clusters involved in remodeling of the extracellular matrix and the cytoskeleton; up-regulation of these genes was suppressed by VSL#3 treatment in particular two days after the first TNBS challenge.

Another set of genes that was up-regulated in the colons of mice with colitis comprised of α-defensins and related antimicrobial peptides. Table 1 shows a list, including the fold-change and p-values, to illustrate the inhibitory effect of VSL#3 on up-regulation of these defensin-related cryptdins. *L. plantarum* was ineffective in this respect.

At several time points dedicated evaluation of genes encoding TLR or associated pathways did not reveal major changes in response to colitis induction and/or probiotics. Yet, as will be discussed below major effects of colitis induction and probiotic treatment concerned inflammatory mechanisms.

Probiotics inhibit the expression of gene transcripts associated with mast cell recruitment and activity

Detailed analysis revealed increased expression of mast cell protease-1, mast cell protease-4, chymase-1, chymase-2, carboxypeptidase A3, and FcεRI in mice with colitis. Figure 3 shows a heat map of expression profiles of these mast cell related genes in mice with colitis compared to healthy controls, as well as expression patterns in mice treated with probiotics. Although not all of these genes were comparably induced by colitis induction, the overall pattern strongly suggested an increase of mast cells in the tissues, which was not observed in mice subjected to treatment with VSL#3 or *L. plantarum*. As will be discussed in the next section these observations were partly reflected by lower numbers of mast cells.

Because local chemokine production is key to the infiltration of the intestine by immune cells, we addressed the expression of a set of chemokines during the course of colitis in this model. We assessed gene expression profiles of mice treated with VSL#3, two and seven days after each TNBS challenge and compared these profiles with vehicle-treated mice. As shown in Figure 4A, genes encoding several chemokines involved in the recruitment of mast

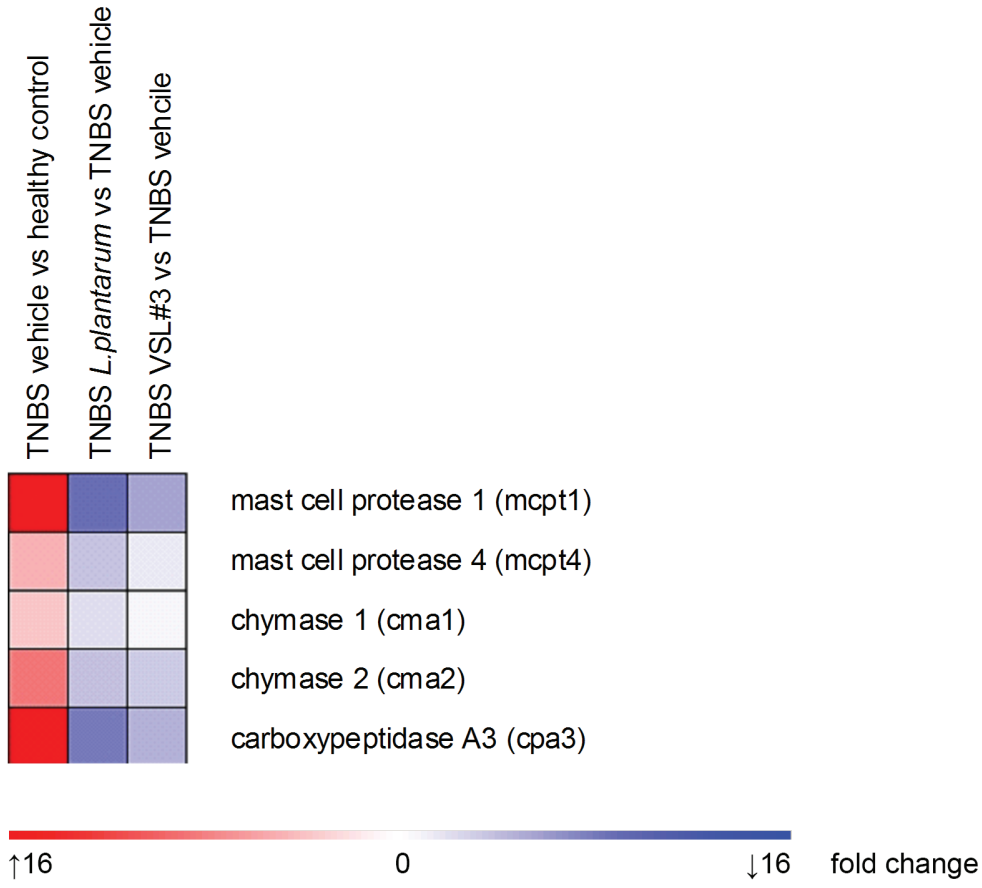

Figure 3.
Heat map of gene expression of mast cell-associated enzymes (red indicates up-regulation, blue indicates down-regulation)

cell progenitors and other inflammatory cells to the intestinal mucosa were up-regulated2 days after each TNBS challenge, but not at 7 days post-challenge. RT-qPCR confirmed the enhanced expression of chemokines after each TNBS-challenge, this analysis also showed that VSL#3 treatment suppressed up-regulation of these chemokines during the first TNBS-challenge (Figure 4B), but not at later time points (data not shown).

Probiotics prevent colitis associated influx of inflammatory cells into colonic (sub) mucosa

As shown in Figure 5A, TNBS-induced colitis was associated with increased infiltration of the lamina propria and the submucosa by mast cells. Although treatment with probiotics resulted

FIGURE 4A

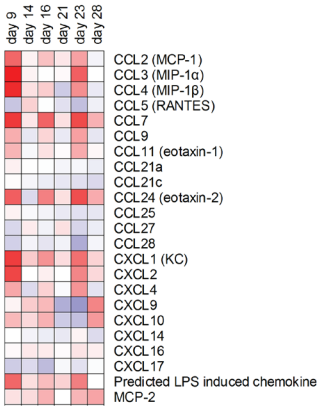

FIGURE 4B

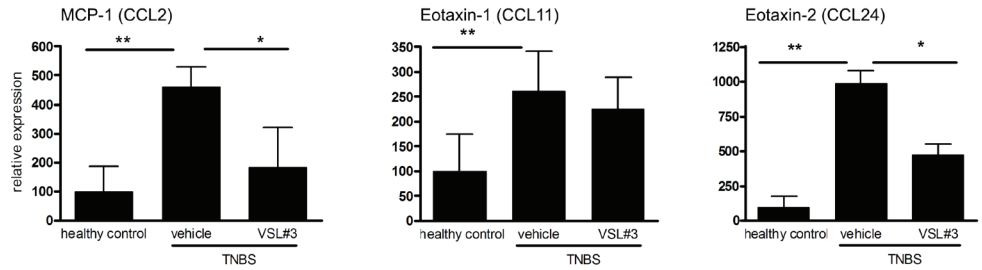

Figure 4.
(A) Chemokine expression in the TNBS colitis model at various time points (B) Effect of VSL#3 treatment on gene expression profiles of chemokines involved in the recruitment of mast cells on day 9.

in a lower expression of mast cell associated enzymes, this was only in part reflected by lower numbers of mast cells, visualized by toluidin blue or specific staining with anti-FcεRI. Also numbers of other inflammatory cells including $CD4^+$ T cells, $CD8^+$ T cells and $CD11b^+$ innate immune cells confirmed as macrophages by F4/80 staining were significantly increased after TNBS-colitis induction. Mice treated with *L. plantarum* or VSL#3 showed significantly lower numbers of these cells (Figure 5B).

To study whether these inflammatory processes were reflected in serum at endpoint, we performed a multiplex analysis and evaluated a panel of 23 cytokines and chemokines. Table 2 displays only those cytokines and chemokines that were modulated in mice treated with probiotics. Colitis induction was associated with increased levels of several (pro)-inflammatory cytokines and chemokines (GM-CSF, IFN-γ, IL-10, IL-17, IL-1β, and MCP-1). Mice treated with VSL#3 showed significantly decreased levels of IFN-γ, IL-17, GM-CSF, IL-1α, IL-1β, MCP-1 and MIP-1α as compared to vehicle controls. In this respect, *L. plantarum* treatment was less effective with a trend towards down-regulation of several of these cytokines and only significant inhibition of IL-17 and IL-3.

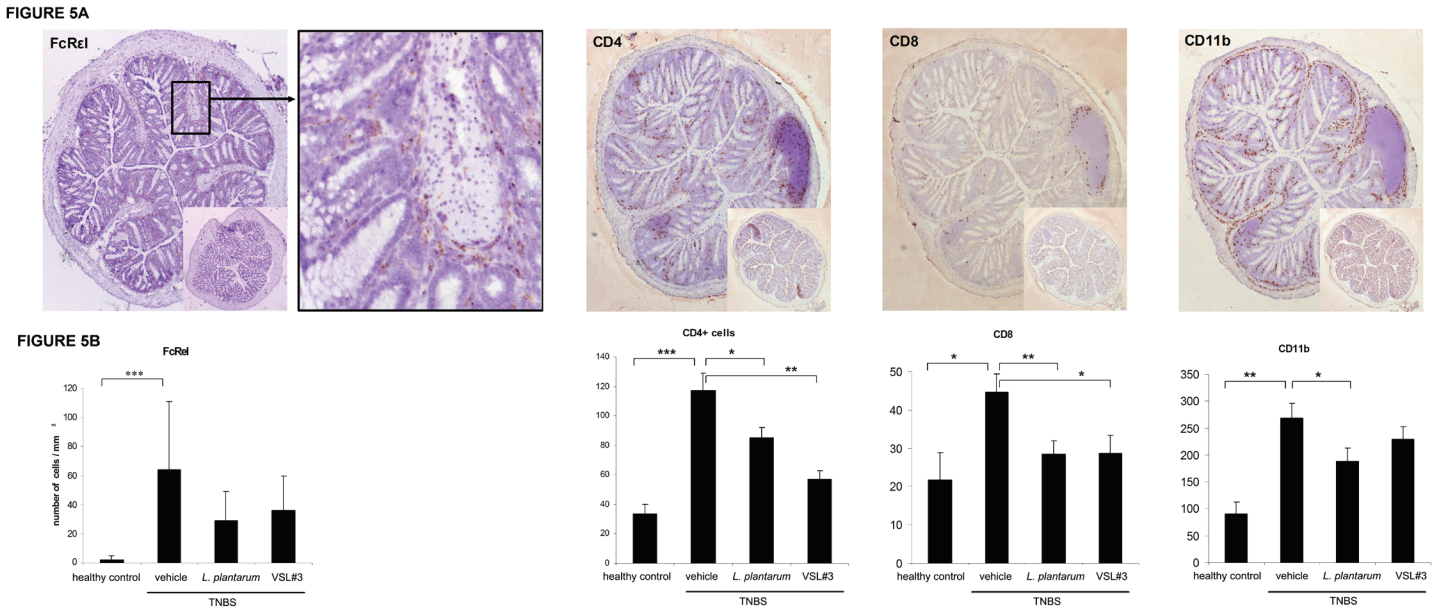

Figure 5.
Probiotics decrease infiltration of lamina propria and submucosa by inflammatory cells. **(A)** Representative section of inflamed colon tissue collected on day 28 and immuno-stained with anti-FcRεI anti-CD4, anti-CD8 or anti-CD11b The inlay shows positive cells in healthy control mice **(B)** Quantification of positive cells was based on 3 non-serial colon sections per mice and normalized against the mucosal area. Bars represent group means for the number of cells on day 28. Mann-Whitney U-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Discussion

Relapsing colitis in BALB/c mice was mimicked by weekly intrarectal administrations of low dose TNBS in ethanol. The model is characterized by the initial production of Th1 cytokines followed by a Th17- like profile after 3 weeks (9). We confirmed increased levels of IFN-γ, IL-17 and several chemokines in serum samples, collected three weeks after the first rectal TNBS challenge, in conjunction with a gradual increase of inflammatory cells in the colon.
Because it has been suggested that probiotics may modulate local immune response by affecting regulatory T cells and/or T cell differentiation(13),we choose for this intermediate time-point with developing pathology to evaluate the effects of probiotic treatment. Likewise, we selected a time-point of 7 days after the third intrarectal challenge, to avoid the acute phase directly following each rectal challenge. Therefore, our results reflect effects of probiotics on a gradually developing process of inflammation, characterized by the influx of T cells and macrophages with moderate pathology as an aspect of IBD, rather than effects on disease with high severity.
In the present study, we have shown that this model is sensitive to prophylactic treatment with probiotics, evident from less intestinal inflammation and normalized colonic gene expression profiles. Beneficial effects of probiotics were previously reported for *L. plantarum* NCIBM8826 and VSL#3 in spontaneous (14;15) and chemical induced (16-18) models of experimental colitis. Here, we confirm that probiotics significantly affect the host, rendering the mice largely resistant to the development of disease in response to multiple challenges with TNBS. Moreover, we substantiated these effects by genome wide mRNA profiling. In our experiments, both *L. plantarum* and VSL#3attenuated the development of clinical features of colitis, with favorable effects on gain of bodyweight and slight improvement of colon morphology.
Both in UC and CD, the colonic mucosa is infiltrated by neutrophils, macrophages, and lymphocytes. This feature of human IBD is reflected in the relapsing TNBS colitis model by the infiltration of $CD4^+$, $CD8^+$ T cells, macrophages and mast cells in the intestinal mucosa after 3 repetitive colitis inductions. Influx of these inflammatory cells was reduced in mice treated with either *L. plantarum* or VSL#3, and this might be due to local down regulation of chemokines. Indeed, various chemokines key to the recruitment of inflammatory cells were less up-regulated after the first TNBS challenge, in mice treated with VSL#3. At endpoint, diminished levels of MIP-1α, G-CSF, GM-CSF, and MCP-1were demonstrated in serum. The beneficial effects of probiotics in this study were also evident from an overall reduction of inflammatory serum cytokines. Seven days after the last TNBS challenge untreated mice with colitis showed increased levels of several cytokines, including IL-1α, IL-1β, IFN-γ, MIP-1α and IL-17. These cytokines are suggestive for an involvement of macrophages and a mixed Th1/Th17 population (19;20)(21). These observations are of importance, since CD has

recently been characterized as a Th1/Th17 driven inflammatory disease (22). Importantly, also these cytokines were significantly lower in serum of mice treated with VSL#3.

There is strong evidence for an involvement of mast cells in the pathogenesis of IBD (23). This is of interest since the relapsing colitis model involves a mast cell component, evident from progressively elevated mRNA levels of transcripts encoding mast cells (Kremer, et al 2012). However, the exact role of mast cells in intestinal inflammation is topic of debate. Mast cells were shown to protect from colitis by enhancing the barrier function of epithelial cells and by limiting spontaneous development of colitis in susceptible IL-10$^{-/-}$ mice(24). However, mast cells have also been shown to increase epithelial barrier permeability (25) and the recruitment of inflammatory cells to the site of infection by the release of cytokines and several pro-inflammatory mediators like trypases and chymases (26;27). In the TNBS colitis model the pathology is mast cell dependent, since colitis could not be induced in mast cell deficient mice(28). Furthermore mast cell stabilizers (29;30) reduce symptoms of colitis. In our study, VSL#3 was able to reduce up-regulation of mast cell related genes in acute phases of colitis. During the first acute phase, following the first rectal challenge, this was accompanied by suppression of up-regulation of MCP-1 (CCL2), Eotaxin-1 (CCL11), and Eotaxin-2(CCL24). These chemokines have been implicated in the recruitment of mast cells (31;32) and can be produced by intestinal epithelia cells or leukocytes in the colonic mucosa. Recently, studies in the DSS colitis model demonstrated that Ly6C^{high}CCR2^{+} inflammatory macrophage are the major source of CCL11(33). It might therefore be that the anti-inflammatory effects of VSL#3 are partly due to a direct effect on these cells, thereby limiting mast cell recruitment. Strategies to modulate mast cell infiltration may bea therapeutic strategy in human IBD, since increased numbers of mast cells are found in both inflamed and non-inflamed tissue in UC and CD patients (34).

Although several genes downstream TLR, including cytokines and chemokines were modulated by probiotics, we did not observe differential expression of all 13 known mouse TLR. Also Metacore analysis did not reveal significant enrichment of genes involved in TLR signaling. Possibly, such effects require a more dedicated analysis of specific cell type rather than a heterogeneous population of cells as present in the intestinal mucosa.

In view of the anti-inflammatory effects of VSL#3 treatment, it was surprising to observe that VSL#3 reduced colitis associated upregulation of α-defensin mRNA levels in the inflamed colon. α-defensins are predominant antimicrobial factors involved in the host defense against bacteria, fungi, protean, and viruses(35). In the intestines these anti-microbial peptide are mainly produced by Paneth cells. Until recently, these α-defensins were not considered to be produced in the colon of mice(36;37). However, this idea was based on normal colon in homeostatic conditions and more recently α-defensins producing Paneth cells were described to play a role under inflammatory conditions in the colon(38). Our observations are of interest because an increased expression of human defensin A5 and A6 (DEFA5

&DEFA6) was also observed in the colon of (pediatric) IBD patients(39;40), which was largely due toPaneth cell metaplasia. It has been suggested that the anti-microbial peptides are produced as a mucosal defense mechanism to counteract a bacterial attack, induced by tissue damage. Therefore, we speculate that reduced α-defensin gene expression in VSL#3 treated mice, is due to less excessive inflammation and consequently less involvement of metaplastic Paneth cells.

The recurrent TNBS colitis model comprises several features of IBD and due to the cytokine profiles and histopathological features during the early stages it has similarities with Crohn's disease. These results may be surprising because in human clinical trials probiotics have been shown effective in prolonging the remission phase of ulcerative colitis, but not in Crohns' disease (41). However, it should be taken into account that the recurrent TNBS model is rather a more generalized model of colitis, with shifting cytokine profiles. Moreover, it should be taken into account that our studies reflect the prophylactic activity of probiotic treatment whereas in patients treatment was initiated during ongoing disease. In view of our data it is tempting to speculate that the primary effect of probiotics is to down-regulate chemokines and thereby control the severity of inflammation. Likewise, evaluation of probiotic treatment initiated after the induction of colitis may help to reveal which mechanisms are relevant to the efficacy of probiotics in this model.

Further studies addressing the effect of probiotics on local chemokine expression and underlying mechanisms in colitis may open new avenues for the treatment of IBD

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Chapter 4

Human APOC1 transgenic mice with atopic dermatitis display intestinal inflammation and are sensitive to probiotic bacteria

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Abstract

Genetic predisposition and environmental factors including the gut microbiota have been suggested as major factors in the development and progression of atopic dermatitis. Hyperlipidemic human APOC1+/+ transgenic mice display many features of human atopic dermatitis, along with a disturbed skin barrier function. Cytokine analysis of serum shows an increase of various pro-inflammatory cytokines, but lower levels of IFN-γ. These mice also display aspects of colitis evident from macroscopic and histological abnormalities. Genome-wide transcriptome analysis of the intestine shows upregulation of several genes associated with mast cells and eosinophils and this observation was confirmed by demonstrating increased numbers of IgE+ and FcRε+ mast cells in the colon and in the skin. Oral treatment with *L. plantarum* resulted in decreased numbers of mast cells in the colon and less severe skin pathology, suggesting that modulation of intestinal immune homeostasis contributes to the suppression of atopic dermatitis.

Introduction

Atopic Dermatitis (AD) is a chronic relapsing skin disease characterized by intense pruritus and the development of inflammatory lesions[1].The exact etiology, pathophysiology and pathogenesis of AD are not fully understood, although multiple genetic and environmental factors have been implicated [2]. The incidence of AD in the Western world has dramatically increased during the past decades, currently affecting 20% of young schoolchildren and 6% of the total population[3]. Atopic diseases, such as rhinitis, asthma and AD, are the result of an inflammatory reaction triggered by type 2 T helper (Th2) cell-mediated immune responses against 'innocuous' antigens (allergens) in susceptible individuals [4]. Environmental changes associated with a Western lifestyle are thought to be involved in the increased incidence of atopic diseases. According to the hygiene hypothesis [5,6] reduced exposure to pathogenic and nonpathogenic microbes with concomitant decreased cellular immunity enables enhanced Th2 responses [7].

The intestinal microbiota plays a key role in immune homeostasis and distinct enterotypes may be associated with established and developing allergic disease [8-11].Epidemiological data showed that the intestinal flora of atopic children differs from that in healthy children [9]. Interestingly, *Lactobacillus* and *Bifidobacteriumspp* are more frequently found in the intestinal flora of non-allergic children compared with allergic children [12]. Accordingly, probiotic bacteria have been suggested for the treatment of AD[13]. Both animal and clinical studies have shown promising effects of probiotic bacteria by modulating allergic responses [14,15].Although the mode of action of probiotic bacteria is still unclear, part of their beneficial effects in AD patients may be explained by the induction of regulatory cytokines [16].

Interestingly, *L. rhamnosus* has been shown to improve barrier integrity of the small intestine in children with AD [17]. This observation is in line with the notion that the intestinal microbiota and probiotic bacteria may exert effects by contributing to intestinal immune homeostasis and barrier function [18].

Recently, we demonstrated that probiotic treatment inhibits the induction of colitis, largely by reducing the local pro-inflammatory milieu [19]. Because the gut-associated mucosal immunesystem is considered as a key regulator of inflammatory diseases [20], we wished to evaluate intestinal inflammation in human APOC1+/+ transgenic mice and study effects of treatment with probiotic bacteria .Previously, we demonstrated that these mice have a disturbed lipid profile [21] and develop skin pathology that resembles several aspects of human atopic dermatitis [22,23]. As compared to other mouse models of atopic dermatitis, the APOC1 mouse model has the advantage that it combines skin inflammation with a disturbed skin barrier integrity [24]. In the present study, we show that these mice with a genetic predisposition to develop strong Th1 immunity, show Th2-type inflammation of the colon. Treatment with *L. plantarum* not only reduced intestinal inflammation but also ameliorated features of AD in these mice.

Results

Human APOC1 transgenic mice with atopic dermatitis display changed colon morphology

In this study we wished to evaluate the integrity of the intestine in human APOC1+/+ transgenic mice. Previously, we reported that these mice show hyperlipidemia and the development of AD [21,22]. Accordingly, the mice in this study showed increased levels of triglycerides and cholesterol compared to their heterozygous littermates (Figure 1A). Furthermore, lipoprotein fractionation by FPLC on plasma of 4-hour fasted mice showed that the total cholesterol was mainly composed of VLDL (Figure 1B). These mice displayed a variable severity of AD, based

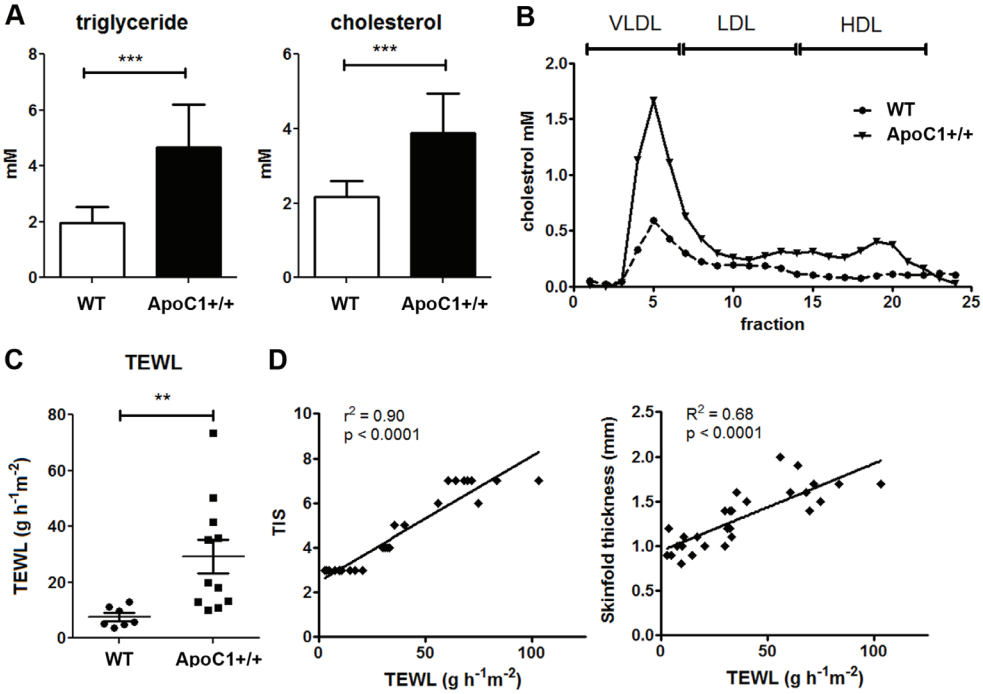


Figure 1. Disturbed serum lipid levels and symptoms of atopic dermatitis in human APOC1 transgenic mice.

(A) Plasma triglycerides and total cholesterol are elevated in APOC1 homozygous (APOC1 +/+)

(B) 4-hour fasted mice were bled, and plasma was pooled per group (n=8-12 / group). The distribution of cholesterol over the individual lipoproteins was determined after separation by fast protein liquid chromatography.

(C) Increased transepidermal water loss (TEWL) was observed in the upper dorsal area of 15-week-old APOC1+/+. Values are means ± SD (n = 8-12 per group. *p <0.05, ** p<0.01, ***P<0.001)

(D) In a separate cohort of 28 APOC1 +/+ mice (♂/♀ n = 28), TEWL was correlated to the severity of dermatitis according to the TIS score as well as to skinfold thickness.

on three parameters: 1) the clinical appearance2) thickening of skin and 3) trans-epidermal water loss (TEWL). APOC1+/+ mice displayed increased TEWL(Figure1C),indicative for loss of integrity of the skin barrier. As shown in Figure 1D, these TEWL-values were highly correlated both with the three-item severity score (r^2= 0.90, p<0.0001) and the thickness of the skin (r^2= 0.68, p<0.0001). No obvious correlation between serum lipids and AD parameters were observed at endpoint.

Apart from the skin pathology, these APOC1+/+ mice displayed intestinal abnormalities. As shown in Figure 2A, stool in APOC1+/+ was soft as compared to wild-type animals. In the distal part of the colon soft feces was observed in >90% of APOC1+/+ mice (n=16), but not in wild-type mice. These abnormalities in stool consistency were accompanied by macroscopic and histopathological changes of the colon. APOC1+/+ had an increased colon length (Figure 2B) and showed increased thickening of the distal colon (Figure 2C).Furthermore, APOC1+/+ mice displayed increased inflammation of the colon (Figure 2D).

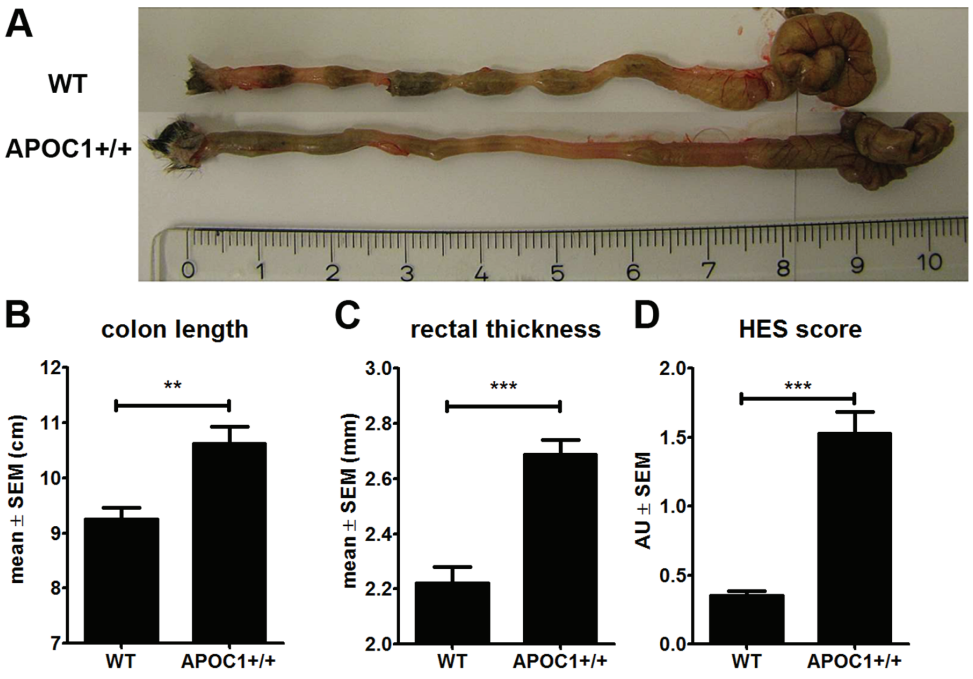
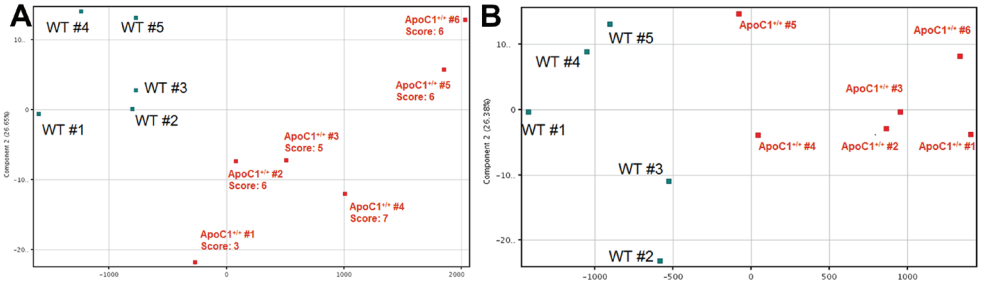


Figure 2. Changes in colon morphology of human APOC1 transgenic mice
Overexpression of human APOC1 is associated with soft feces in the distal part of the colon (A). Colons of these mice show a significantly increased length (B), thickness (C) and histological score (D). Bars represent group averages, error bars represent SD. Mann-Whitney U-test; * p<0.05, ** p<0.01, *** p<0.001.

Genome-wide gene expression profiling of the colon ofAPOC1 transgenic mice

To gain further insight into the abnormalities observed in the colon ofAPOC1 +/+ mice, we evaluated genome-wide gene expression in colon and small intestine. Principal Component Analysis (PCA) was performed using all 26,000 probes on the microarray. This unsupervised approach separated the APOC1+/+ mice from the wild-type mice. Figures 3A and 3B show PCA plots illustrating differential gene expression patterns between APOC1+/+ and wild-type mice for colon and small intestine. Statistical analysis between ApoC1+/+ and wild-type mice identified 1,252 genes and 1,001 genes that were differentially expressed (fold change >1.5, pBayes< 0.05) in the colon and small intestine, respectively. To further elucidate the processes associated with these differentially expressed genes, MetaCore™ software was applied to identify enriched process networks. The ten most significant processes are depicted in Figure 3C. Five out of 10 and 3 out of ten of these processes were related to immune function in small intestine and colon, respectively. In view of the observed changes in colon morphology, we further focused on processes in the colon. Detailed analysis of immune function revealed elevated levels of mast cell related transcripts including cpa3,cma2, mcpt-1, mcpt-4, and mcpt-9 in colons of APOC1+/+ mice compared to wild-type mice (Figure 4A). Immunohistochemical staining of FcεRI+ and IgE+ cells confirmed the presence of large numbers of mast cells in the lamina propria of APOC1+/+ mice as shown in Figure 4B and 4C, respectively. The expression of several genes encoding pro-inflammatory chemokines



C

Small intestine Networks	ratio#	p-value	FDR
Muscle contraction	25/173	6,6E-07	1,0E-04
Development_Skeletal muscle development	20/144	1,6E-05	1,1E-03
Cytoskeleton_Regulation of cytoskeleton rearrangement	23/183	2,0E-05	1,1E-03
Cell adhesion_Leucocyte chemotaxis	24/205	4,4E-05	1,7E-03
Immune response_TCR signaling	21/174	8,5E-05	2,7E-03
Cell adhesion_Cell junctions	19/162	2,7E-04	7,1E-03
Reproduction_Feeding and Neurohormone signaling	22/211	4,9E-04	1,1E-02
Cytoskeleton_Actin filaments	17/176	4,7E-03	9,2E-02
Proliferation_Lymphocyte proliferation	19/209	5,5E-03	9,7E-02
Cell adhesion_Integrin-mediated cell-matrix adhesion	19/214	7,1E-03	1,1E-01

Colon Networks	ratio#	p-value	FDR
Proteolysis_Connective tissue degradation	20/119	8,9E-05	1,4E-02
Proteolysis_ECM remodeling	15/85	3,9E-04	3,1E-02
Development_Regulation of angiogenesis	28/223	7,1E-04	3,1E-02
Inflammation_Interferon signaling	17/110	8,1E-04	3,1E-02
Muscle contraction	23/173	9,6E-04	3,1E-02
Development_Neurogenesis_Axonal guidance	28/230	1,2E-03	3,1E-02
Cell adhesion_Amyloid proteins	24/195	2,2E-03	4,4E-02
Inflammation_IFN-gamma signaling	16/110	2,2E-03	4,4E-02
Development_Skeletal muscle development	19/144	2,8E-03	5,0E-02
Reproduction_Progesterone signaling	25/213	3,4E-03	5,2E-02

Figure 3. Genome wide gene expression profiling of the intestines of APOC1 transgenic mice. Two-dimensional visualization of principal component analysis (PCA) constructed with all genes on the microarray, discriminating APOC1+/+ mice form wild-type C57BL/6 mice. Each dot represents the expression profile of an individual mouse for colon (3A) and small intestine (3B). Top ten enriched process networks are depicted (3C).

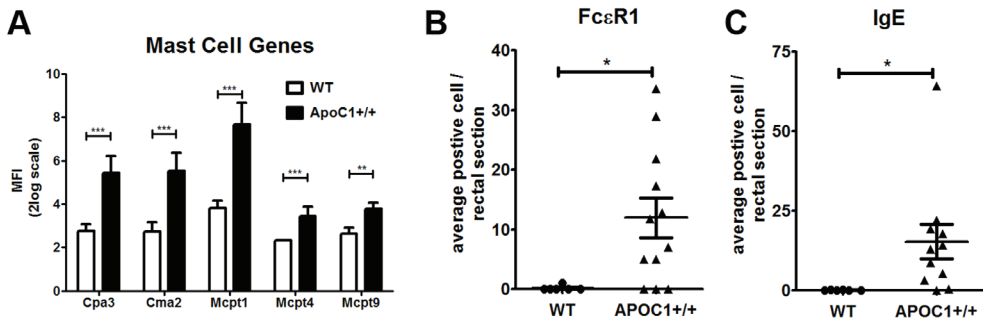


Figure 4. APOC1 transgenic mice have increased levels of mast cells in the colonic mucosa
(A) Gene expression levels of mast cell-associated enzymes in wild-type C57BL/6 and APOC1+/+ mice. Mean ± SEM was based on 6 mice / group
(B) Colon tissue was immunostained with anti-IgE, anti-FcεR1 and counterstained with hematoxylin. Quantification of positive cells was based on 4-5 non-serial colon sections. Results are depicted as mean ± SEM for all individual mice per group. Statistical significance was calculated using the Mann-Whitney U test. *, P < 0.05; **, P < 0.01.

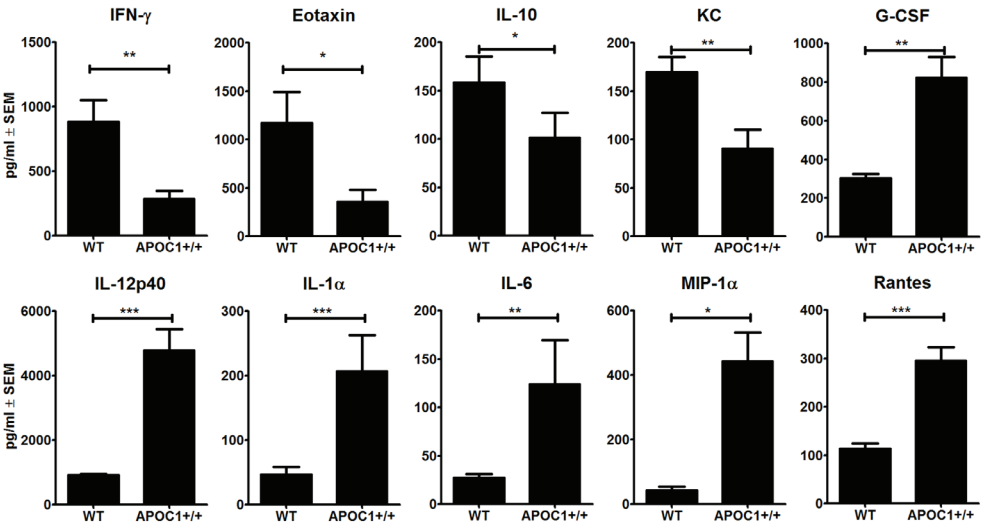


Figure 5. Reduced IFN-γ and IL-10 levels in the serum of APOC1+/+ mice.
Serum samples of APOC1+/+ and C57BL/5 mice were evaluated using a 23-plex bead assay as indicated in Material & Methods. Only cytokines that showed significant differences between the groups are depicted.
Bars represent group averages, error bars represent SD. Mann-Whitney U-test; * p<0.05, ** p<0.01, *** p<0.001

associated with the recruitment of mast cells [25] were also up-regulated in APOC1+/+ mice compared to wild-type controls. These include *Ccl-5, Ccl-11 and CXCL-14;* enhanced mRNA expression of these chemokines was accompanied by increased expression of eosinophil associated transcripts like *RNAse2* and *Ear2, -8, 10* (see supplementary Table). In contrast, *Cxcl-9* and *Cxcl-10* as well as IL-18 were down-regulated in APOC1+/+ mice compared to wild-type mice.

Several pro-inflammatory cytokines, including IL-12p40, IL-6 and IL-1α, were increased in serum of APOC1+/+ mice (Figure 5). Th2 cytokines (IL-4, IL-5, IL-13) were below the detection limit; however, IFN-γ and IL-10 levels were lower ($p<0.01$ and $p<0.05$, respectively) in APOC1+/+ mice.

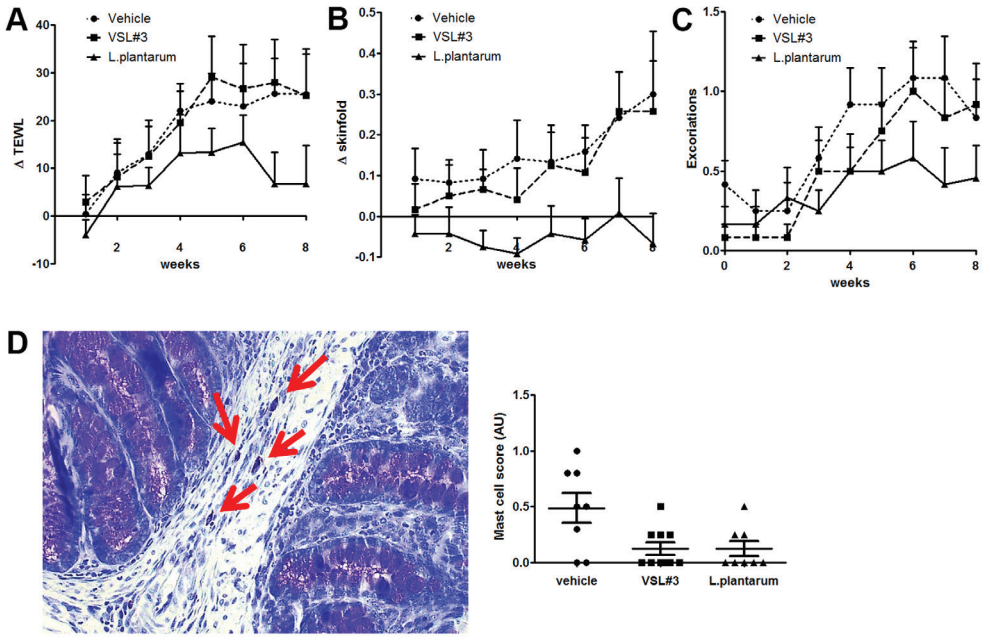


Figure 6. Oral treatment of APOC1+/+ mice with probiotic bacteria results in decreased inflammation of colon and skin.

APOC1+/+ mice (12 mice per group) were treated from an age of 8 weeks onwards and sacrificed after 8 weeks of treatment with vehicle,VSL#3 or L. plantarum. Trans-epidermal water loss (A), skinfold thickness (B) and excoriations (C) were evaluated during the treatment period for each individual mouse compared to baseline levels. Repeated measurement ANOVA, *$p < 0.05$, **$p < 0.01$. After sacrifice, colons were histological evaluated after staining with toluidine blue and assessed in a semi-quantitative fashion(D). Results are depicted as mean ± SEM. *Mann-Whitney U-test, $p < 0.05$.

Decrease inflammation of colon and atopic dermatitis in APOC1+/+ mice treated with L. plantarum

Recently, we demonstrated that probiotic treatment affects intestinal gene expression and renders BALB/c mice resistant to the induction of colitis [19]. We were therefore interested whether oral administration of probiotics would have a favorable effect on the development of intestinal and skin inflammation. Eight-week-old APOC1+/+ mice with established symptoms of AD were randomized over 3 groups (12 mice / group) and treated with VSL#3*, L. plantarum* or vehicle during a period of 8 weeks. As shown in Figure 6A, *L. plantarum* treatment delayed the loss of the skin barrier integrity as measured by TEWL (repeated-measures ANOVA $P < 0.001$). Furthermore, *L. plantarum* treated mice did not show progression of skin thickening, in contrast to VSL#3, which was ineffective in this respect (RM ANOVA $P < 0.001$) (Figure 6B). Although *L. plantarum* treatment did not affect the three-item severity score, combining three different parameters, it showed a favorable effect on the development of excoriations as shown in Figure 6C (repeated-measures ANOVA $P < 0.01$).

Moreover, mice treated with VSL#3 or *L. plantarum* displayed less inflammation of colon, evident from decreased infiltration by mast cells as shown in Figure 6D.

Discussion

The largest body of immune cells is found in the gastro-intestinal tract and referred as gut-associated lymphoid tissue (GALT). The GALT is continuously exposed to intestinal bacteria, which are recognized by intestinal epithelial cells and dendritic cells, thereby shaping the immune system [26,27]. An uncontrolled immune response against the commensal microbiota has been linked to several (chronic) intestinal disorders, such as Crohn's disease, ulcerative colitis and celiac disease [28,29]. Moreover, perturbations in the gut microbiota have been linked to dysregulated immune responses and inflammatory diseases at peripheral tissue sites [30].A strong association between the development of allergic disorders like asthma and atopy and the composition of the fecal microbiota of infants has been reported [31,32]. In this study, we showed that human APOC1+/+ transgenic mice that show human AD-like features also show inflammation of colon and substantial changes in intestinal gene expression. Furthermore, oral administration of *L. plantarum* resulted in amelioration of both colon and skin pathology in these mice.

Genome wide associated studies identified only one common gene (C11Orf30) on susceptible loci in AD and inflammatory bowel disease (IBD) patients [33,34]. However, epidemiological studies showed an increased prevalence of AD and IBD patients [35,36]. Recently, a large 11-year prospective study demonstrated an association between ulcerative colitis - a Th2 driven intestinal inflammation – and AD. Additionally, patients with AD appear to have an increased

intestinal permeability [37][17]. Herein, we showed that AD-prone humanAPOC1+/+ mice have colon abnormalities evident from altered macroscopic features and stool consistency. Unsupervised analysis of the transcriptome data set also revealed several genes in the small intestine related to barrier and host defense, such as anti-microbial peptides, that were down-regulated (data not shown). Reports on macroscopic and histological features of the large intestine in patients with atopic dermatitis are scarce. To our knowledge, only one study by Arisawa et al describes pathological examination of the colon of these patients [38]. They concluded that AD patients might have a latent chronic inflammation in the large intestine, marked by increased eosinophilic infiltration in the intestinal mucosa, as compared to healthy individuals [8]. Interestingly, transcriptome analysis of the colon of APOC1+/+ mice also implicated a role for eosinophils and mast cells.

C57BL/6 is a prototypeTh1 mouse strain with high expression of IFN-γ and low levels of IL-4 [39]. However, human APOC1+/+ mice have decreased serum levels of IFN-γ and IL-10, whereas general inflammatory mediators such as IL-12p40, IL-6 and TNF-α were increased. In the colon, expression of *Cxcl-9,Cxcl-10, Il-18* – formally depicted as IFN-γ inducing factor – was decreased in APOC1+/+ compared to wild-type mice controls. These findings together with the infiltration of IgE and FcRε+ cells suggest a Th2-driven inflammation in the colonic mucosa of APOC1+/+ mice.

Epidemiological studies have indicated that the incidence of allergies, including atopic dermatitis, has increased during the past decades in many western countries [40]. This increased disease incidence seems to be associated with a variety of environmental factors: the extended hygiene hypotheses, antibiotic use, lifestyle changes or a western type diet [6,41]. According to this hypothesis, commensal gut bacteria might induce an anti-inflammatory response by inducing regulatory T cells [42]. It might also be that a lack of pro-inflammatory stimuli due to increase hygiene results in a decreased Th1 response and an increased Th2 response causing allergic diseases [43]. Therefore modulating intestinal immune homeostasis by oral administration of selected probiotics have been proposed as a strategy to treat or prevent atopic dermatitis. In this study *L. plantarum* diminished Th2 type inflammation in the colon, i.e. decreased numbers of mast cells, along with favorable effects on aspects of atopic dermatitis. Failure of the probiotic mixture VSL#3 in ameliorating skin pathology, despite favorable effects on colon inflammation, suggests that different strains may act differently in diverse pathological conditions.

In conclusion, our data suggest that over-expression of human APOC1 results in a Th2-type inflammatory response in intestine and skin, despite a Th1-type background. Because inflammation of both tissues is improved by oral administration of probiotic bacteria it is tempting to speculate that ensuring intestinal immune homeostasis contributes to the control of atopic dermatitis.

Materials & methods

Mice

Human APOC1(+/+) transgenic mice were generated on a C57BL/6 background as described previously [21]. Wild-type C57BL/6 mice were purchased from Charles River and housed under the same conditions. All studies were performed in the animal facility of TNO with a 12h light/dark cycle. Mice had free access to standard mouse chow (SSNIFF R/M-H, Bioservices B.V., Uden, the Netherlands) and water. Animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Probiotic treatment

VSL#3 (Ferring Pharmaceuticals, Berkshire, UK) was purchased as a commercially available probiotic mixture containing freeze-dried bacteria (*Bifidobacterium longum, B. breve, B. infantis, Lactobacillus acidophilus, L. plantarum, L. casei, L. bulgaricus,* and *Streptococcus thermophilus*).
L. plantarum NCIMB8826 was grown until mid-exponential growth phase (OD600nm = 1) as described previously [19].

Assessment of dermatitis

Mice were monitored regarding the progression of dermatitis with the use of a Three-Item-Score (TIS) score comprising the following items: 1) scaling, 2) papules and lichenification, 3) excoriations, as described previously [23]. Each of the items were graded from 0 (normal) to 3 (severe), and included in a TIS scale ranging from 0 to 9. Thickness of a fold of skin in the upper dorsal area was measured employing a digital caliper (Mitutoyo, Veenendaal, The Netherlands). TEWL was measured by placing a 12-mm detection probe of a skin evaporative water recorder (Tewameter® TM 300, Courage &, Khazaka, Cologne, Germany) on the upper dorsal skin area of each individual mouse.

Transcriptome analysis

Total RNA was isolated from frozen colon or small intestine using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the instructions of the manufacturer. RNA was treated with DNAse and purified using a nucleospin RNAII Total RNA Isolation kit (Macherey-Nagel, Düren, Germany). RNA integrity was checked employing an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips, according to the instructions of the manufacturer. RNA integrity numbers were above 8.7.
Microarray studies were performed as described previously [44].In short, biotinylated cRNA was prepared using the IlluminaTotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, USA) starting with 500 ng total RNA. Per sample, 750 ng of cRNA was used to hybridize to

the SentrixMouseRef-8 BeadChips (Illumina, Inc., San Diego, CA, USA). Each BeadChip contains eight arrays and each of the arrays harbors 25697 probes. GeneSpringGX 11.0 was used for quantile normalization of the probe-level, background subtracted expression values. Differentially expressed probes were identified using the limma package of the R/Bioconductor project, applying linear models and moderated t-statistics that implement empirical Bayes regularization of standard errors. The statistical analyses were performed through The Remote Analysis Computation for gene Expression data (RACE) suite at http://race.unil.ch [45]. p-values below 0.05 were used as a threshold for significance of the differential expression. Pathway analysis software Metacore (V6.2), a highly curated Web-based application for identification of gene ontology processes in input gene sets, was used to identify biological processes (GeneGo Inc., St. Joseph, MI, USA).

Histological assessment of the colon

Following the assessment of macroscopic parameters, colons were dissected and fragments were fixated in 4% buffered formalin and embedded in paraffin. Sections of 5 μm were stained with hematoxyllin-eosin-saffron. Inflammation was scored in a blinded manner in 4 to 6 non-sequential sections per colon fragment at a 400× magnification, according to a semi-quantitative scoring system with a scale from 0 to 4, based on the presence of inflammatory infiltrates and mucosal damage.

Immunohistochemisty

Colon fragments were fixated in 4% buffered formaldehyde and embedded in paraffin, or embedded in Tissue-Tek and snap-frozen in liquid nitrogen. From paraffin-embedded tissue, 5 μm sections were stained with toluidin blue and counterstained by hematoxylin-eosin, to enable the assessment of mast cells in semi-quantitative fashion. Immunohistochemistry was performed on 6 μm cryo-sections with antibodies specific for IgE (R35-118) and FcεRI (MAR-1) (both obtained from BD Biosciences San Diego, CA, USA). Positive cells were counted at a 400× magnification.

Lipid and lipoprotein analysis

After a 4-hour fasting period, EDTA-plasma was collected. Total plasma cholesterol and triglyceride levels were measured (Roche Diagnostics). Lipoprotein profiles were obtained by FPLC, as described previously [46].

Cytokine and chemokine quantification in serum

Cytokine concentrations in serum obtained at each sacrifice were determined by Multiplex analysis using a Bio-Plex Pro 23-Plex Panel, including IL-1α, -1β, -2, -3, -4, -5,-6, -9, -10, -12(p40), -12(p70), -13, -17, IFN-γ, TNF-α, RANTES, MIP-1α and -1β, MCP-1, KC, G-CSF, GM-CSF, and eotaxin (Bio-Rad Laboratories, Hercules, CA, USA) .

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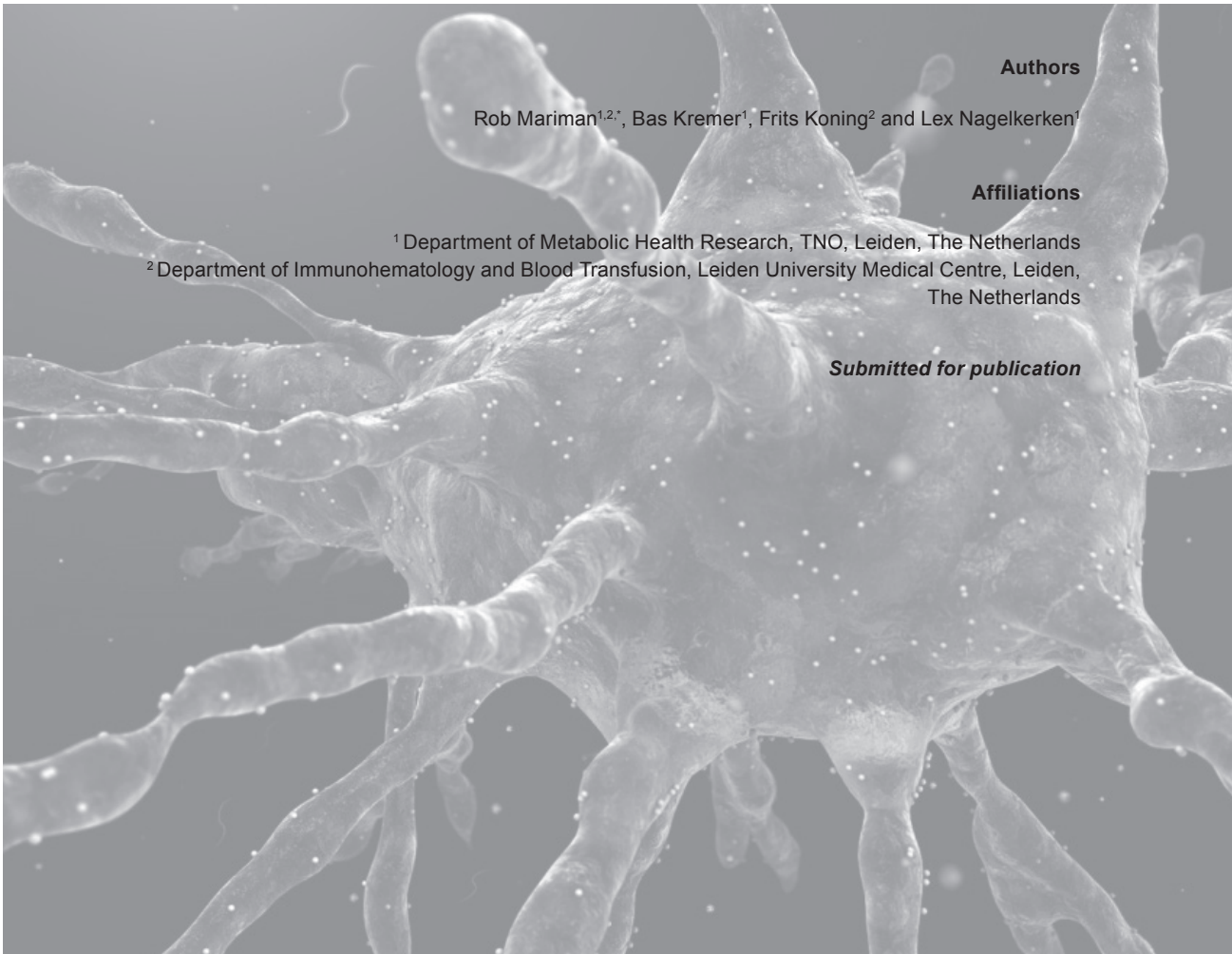
Chapter 5

The probiotic mixture VSL#3 mediates both pro- and anti-inflammatory responses in bone marrow derived dendritic cells from C57BL/6 and BALB/c mice



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Abstract

Probiotic bacteria express a wide range of molecular structures that bind to receptors on innate immune cells and mediate health-promoting effects in the host. We have recently demonstrated in a colitis model that favorable effects of the probiotic mixture VSL#3 may in part be due to suppression of intestinal chemokine expression. To obtain more insight into the underlying mechanisms, we studied modulation of bone marrow derived dendritic cells (BM-DC) from BALB/c (Th2 biased) versus C57BL/6 (Th1 biased) mice. Our data show that VSL#3 differed from pure TLR ligands by inducing higher levels of various cytokines, including IL-12p70, IL-23 and IL-10.Dedicated TLR-arrays were employed to profile mRNA from BM-DC cultured with LPS, VSL#3, or a combination of both. This approach identified (I) a cluster of genes that was up-or down-regulated, irrespective of the stimulus, (II) a cluster of genes that was synergistically up-regulated by LPS and VSL#3 in BM-DC from C57BL/6 mice, but not from BALB/c mice, (III) a cluster of LPS-induced genes that were suppressed by VSL#3, in particular chemokines. These data show therefore that this probiotic mixture has both pro- and anti-inflammatory effects on BM-DC, and suggest that their immune-modulating properties *in vivo* may depend on the genetic background of the host.

Introduction

Dendritic cells (DC) are bone marrow derived antigen-presenting cells capable of inducing protective adaptive immune responses or tolerance. DC are equipped with Toll-like receptors (TLR) and C-type lectin receptors that activate different signalling pathways in response to microorganisms [1].TLR expressed on the cell surface predominantly bind bacterial products, such as lipopeptides and peptidoglycan (TLR1, -2 and -6), lipopolysaccharide (LPS, ligand ofTLR4) and flagellin (TLR5). TLR3, -7, -8 and -9 reside in intracellular organelles and recognize microbial nucleic acids [2]. In the intestinal mucosa, various subsets of DC are in close contact with the intestinal microbiota and continuously migrate from the lamina propria to the mesenteric lymph nodes, ensuring a balance between immunity and tolerance [3]. Lamina propria DC can be divided into two major classes: $CD103^-$ DC induce Th1 and Th17 responses, whereas $CD103^+$ DC rather induce regulatory T cells [4]. Different types of DC develop from bone marrow progenitors and therefore immature bone marrow derived DC (BM-DC) are widely used in *in vitro* studies. Such studies contribute to a better understanding of the mechanism by which microorganisms modulate immune responses *in vivo*. Although not identical to intestinal DC, it has been demonstrated that splenic DC from Th1-prone C57BL/6 mice and Th2-prone BALB/c mice respond differently to microbial stimuli [5-7]and this may in part be explained by differences in TLR expression. BALB/c mice express higher levels of TLR2, -4, and -5 mRNA, whereas C57BL/6 mice express more TLR9 mRNA [8]. In addition, the expression of the macrophage mannose receptor on BM-DC is mouse-strain specific, and this phenomenon may influence antigen-uptake by such cells [9].

Probiotic bacteria modulate DC via surface expression and/or secretion of products which function as ligands for TLR and C-type lectin receptors. The expression of these ligands depends on bacterial species, growth phases and available nutrients, and this explains in part that different species and strains of probiotics vary in their ability to induce cytokines and chemokines. [10][11][12]

Recently, we demonstrated that treatment of BALB/c mice with a mixture of probiotic bacteria (i.e. VSL#3) has profound effects on gene expression in the colons of mice subjected to colitis induction, with favorable effects on the development of disease [13]. However, substantial variability in the response of human subjects to probiotic interventions has been found [14], suggestive for an influence of genetic and environmental factors.

To gain more insight into the influence of genetic background, we choose to study BM-DC from two genetically distinct mouse strains and evaluate their response to the probiotic mixture VSL#3*in vitro*, as compared to ultrapure TLR ligands. Our results, obtained by dedicated gene expression profiling of genes related to TLR-signalling and by studying cytokine production, suggest that VSL#3 may display both pro- and anti-inflammatory effects, dependent on mouse strain.

Materials and methods

Mice

Seven- to eleven–week-old C57BL/6 mice (Charles River, Maastricht, Netherlands) and BALB/c mice (Janvier, St. Berthevin, France) were used in this study. All animal experiments were conducted with the approval of the Institutional Animal Welfare Committee, filed as number DEC2661, in compliance with European Community specifications regarding the use of laboratory animals.

Isolation and culture of BM-DC

BM-DC were isolated as described previously[15], with slight modifications. Briefly, bone marrow was flushed from femur and tibia. Cells were passed through nylon mesh to obtain a single cell suspension. After a single wash step, cells were cultured in RPMI 1640 medium containing 10% foetal bovine serum (Lonza, Verviers, Belgium), 2mM L-glutamine, 100 U/ml streptomycin, 100 µg/ml penicillin (Gibco, San Diego, CA, USA), 50µM β-mercaptoethanol (Sigma Adrich, Zwijndrecht, The Netherlands) in the presence of 20 ng/ml recombinant mGM-CSF (Peprotech, Rocky Hill, NJ, USA). Cells were cultured (10^6 cells/ml) at 37°C. Twothirds of the culture medium were refreshed on days 3 and 6. After 8 days of culture non-adherent and loosely adherent BM-DC were collected and used for stimulation experiments. Viability of cells (>95%) was assessed by trypan blue dye exclusion. Cells were cultured at a cell density of 10^6/ml and stimulated with TLR-ligands and/or probiotic bacteria at concentrations indicated in the figure legends.

Reagents

Pam_3CSK_4, poly I:C,LPS *E. coli K12*, Flagellin *S.typhimurium, CpG*ODN1826, Imiquimod and Peptidoglycan from *E. coli* 0111:B4were purchased from Invivogen (San Diego, CA, USA). All of these ultra-pure TLR ligands were endotoxin free(<0.001 endotoxin units/µg), except for peptidoglycan which contained <125 endotoxin units /ml.

VSL#3, a mixture containing freeze-dried *B.longum, B.breve,B. infantis, L.acidophilus, L.plantarum, L.casei, L.bulgaricus and S.thermophilus*, was purchased fromFerring Pharmaceuticals (Berkshire, UK). *L. plantarum* NCIMB8826and*B.animalis ssp. lactis*BB-82 weregrown at 37 °C in Mann-Rogusa Sharpe (MRS) broth (Scharlau Chemie, Barcelona, Spain) until mid-exponential density (OD_{600nm} = 1). Bacteria were washed twice in phosphate-buffered saline (PBS, pH 7.4). Lyophilized bacteria were prepared by freezing bacterial pellets (-80°C) before overnight lyophilisation in a freeze-dryer under vacuum (40 mBar). Lyophilised bacteria were stored at -20°C until use.

RNA isolation and RT-qPCR

Total RNA from 10^6 cells was isolated using an RNAEasy Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Five hundredng RNA werereverse transcribed into single stranded cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems (AB), Carlsbad, CaliforniaUSA) and incubated for 60 minutes at 37°C and 5 minutes at 95°C. cDNA was amplified employing a 7500 Fast Thermal cycler (AB, USA) using SYBRgreen PCR master mix (AB, USA). Thermal cycling parameters consisted of 1' at 50°C, 10' at 95°C, followed by 40 cycles of 15'' at 95°C s and 1'at 60°C. Primer pairs were for *Il12p35*: 5′-CTG GAG CAT CCG AAT TGC A-3′ (sense, S) and 5′-CAT CCT CTG AGA TTT GAC GCT TT-3′ (anti-sense, AS); *Il23p19:* 5′-GGC AAC TTG GAC CTG AGG AG-3′ (S) and 5′-CAT GGG CTC TCG GTC CAT AG-3′ (AS); *Cxcl9*: 5′-CCT AGT GAT AAG GAA TGC ACG ATG-3′ (S) and 5′-CTA GGC AGG TTT GAT CTC CGT TC-3′ (AS); *Cxcl10*: 5′-ATC ATC CCT GCG AGC CTA TCC T-3′ (S) and 5′-GAC CTT TTT TGG CTA AAC GCT TTC-3′ (AS); *B2m:* 5′- acc gtg aaa aga tga tga ccc ag-3′ (S) and 5′- agc ctg gat ggc tac gta ca-3′ (AS);); *Il12p40*: 5′-GGA AGC ACG GCA GCA GAA TA-3′ (S) and 5′-AAC TTG AGG GAG AAG TAG GAA TGG-3′ (AS). Gene expression levels were quantified according to the formula: $2^{-(Cti-Cta)}$ where C_{ti} is the cycle threshold of the gene of interest and C_{ta} is the cycle threshold value for β-actin.

PCR array

Expression of genes involved in TLR pathways was studied by using the RT^2-ProfilerPCR Array (Mouse TLR-Signaling Pathway) from SABiosciences (Frederick, MD, USA).This array combines the quantitative performance of SYBR Green-based real-time PCR with the multiplegene profiling capabilities of a microarray. 96-well plates containing gene-specific primer sets for 84 relevant TLR pathway genes, 5 housekeeping genes, and 2 negative controls were used. For each experimental condition, RNA was isolated as described above. Gene expression was normalized to internal controls (housekeeping genes) to determine the fold change in gene expression between test and control samples by $\Delta\Delta C_t$ (SA Bioscience).

Cytokine/chemokine analysis

Cytokines were measured in the supernatants of BM-DC cultures 24 hours after cell stimulation using commercially available ELISA kits for IL-12p70, IL-12p40, IL-23p19, IL-10, IL-6, and TNF-α (Ebioscience, San Diego, CA, USA). Chemokines (CXCL-9 and CXCL-10) were quantified using a multiplex immunoassay (Invitrogen). Assays were performed according to the manufacturer's instructions.

Flowcytometryanalysis

BM-DC were phenotypically characterized before and after 24 hours of stimulation. Cells were collected and washed with PBS. 2 x 10^5 cells were incubated (30 min, 4°C, protected

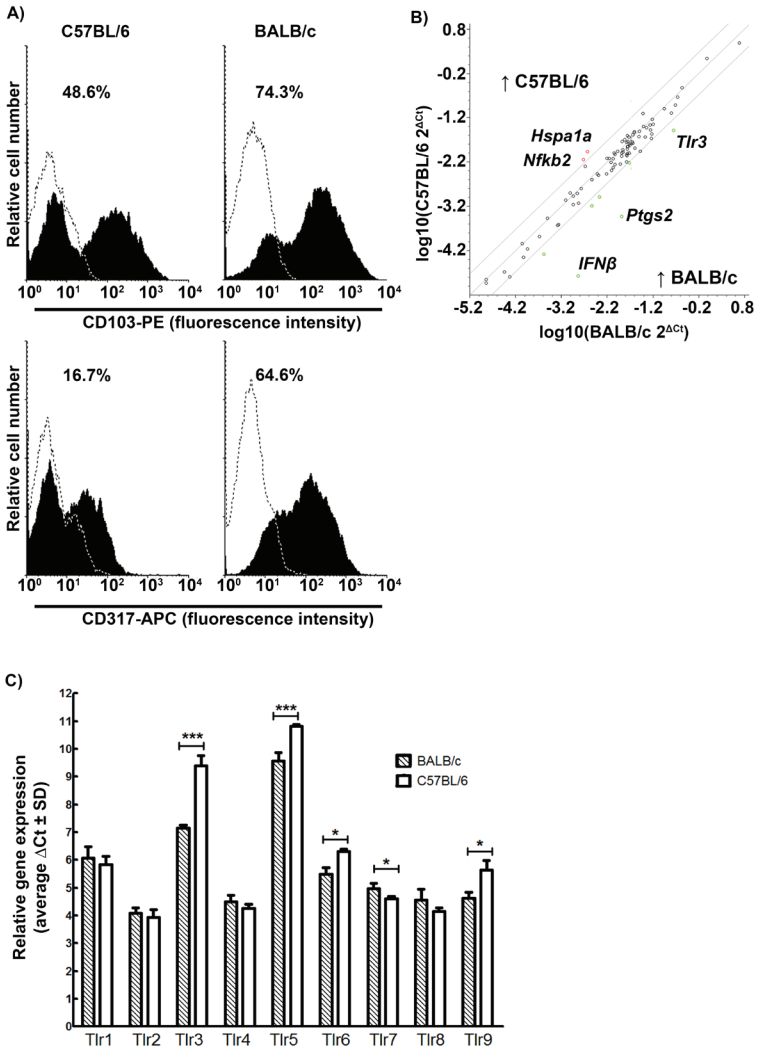

Figure 1. Flowcytometry and gene expression profiling reveals phenotypic differences between BM-DC from C57BL/6 and BALB/c mice

A) Immature BM-DC generated in the presence of GM-CSF were stained with PE-labelled anti-CD103 and APC-labelled anti-CD317 and evaluated by flowcytometry. Filled histograms show the fluorescence intensity for each marker. Dotted lines reflect staining with isotype control antibodies. The percentage of cells expressing these markers is indicated in each panel.

B) mRNA isolated from immature BM-DC was used to evaluate the expression of genes involved in TLR signaling. Results (i.e. an algorithm expressing Ct of the gene of interest relative to the Ct of the housekeeping gene) for C57BL/6 BM-DC (Y-axis) were plotted against results for BALB/c BM-DC (X-axis). Genes that showed at least a 3-fold difference in expression between C57BL/6 or BALB/c BM-DC are indicated.

C) Differences in TLR gene expression between BM-DC from C57BL/6 and BALB/c mice. mRNA isolated from immature BM-DC was amplified by quantitative RT-PCR and Ct-values for each transcript were normalized to a panel of six housekeeping genes. These normalized Ct values are inversely correlated to the level of mRNA expression. Bars represent the normalized Ct values ± SD for BM-DC from 3 individual mice. Student's t-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

from light) with monoclonal antibodies. The following dye-conjugated antibodies CD11c-PerCP/Cy5.5 (N418), CD86-FITC (B7-2GL-1), CD80-Pacific Blue (16-10A1), MHCII-PE (M5/114.15.2), CD317-APC (927), CD103-PE(2E7) were purchased fromBD Biosciences (San Diego, CA, USA). Flowcytometric analyses were performed with a fluorescence-activated cell sorter (FACSCanto II, BD Biosciences) and analyzed with FACSDiva Software 6.1.2.Gating of positive cells was based on the results obtained with isotype control antibodies.

Statistical analysis

Statistical analyses were performed with the Mann-Whitney U-test or Student's *t* test if the samples passed the normality analyses by using RT² Profiler™ PCR Array Data Analysis.

Results

BALB/c and C57BL/6 bone marrow progenitors differentiate into phenotypically distinct DC

BM-DC from BALB/c and C57BL/6 mice were generated by 8 days of culture under identical conditions in the presence of GM-CSF. As shown in Figure 1A, BM-DC from BALB/c mice showed a higher expression of CD103 and CD317 as compared to BM-DC from C57BL/6mice. Moreover, gene expression profiling of transcripts involved in TLR-signalling showed higher expression of*Tlr3*and *Ifnb* in cells from BALB/c mice (Figure 1B).BM-DC from BALB/c mice also showed significantly higher levels of mRNA encoding TLR5, -6 and -9, as compared to BM-DC from C57BL/6 mice (Figure 1C).

Before studying the response of these cells to probiotic bacteria we first evaluated their response to ultrapure TLR ligands. Flowcytometric analysis of the stimulated BM-DC confirmed similar up-regulation of CD80, CD86 and MHC class II expression, regardless of mouse strain or TLR-ligand (data not shown). However, BM-DC from BALB/c and C57BL/6mice showed a different cytokine response to several TLR-ligands, as shown in supportive Figure 1.In particular, the IL-12p70 production in response to LPS or CpG was higher in C57BL/6 BM-DC. These data indicate that the genetic background strongly influences the phenotype and response of BM-DC to pure TLR ligands.

The probiotic mixture VSL#3 and LPS synergistically induce IL-12p70 and IL-23 production in BM-DC from C57BL/6mice

Probiotic bacteria are far more complex than ultrapure TLR ligands and therefore we studied to what extent the genetic background of BM-DC would influence their response to VSL#3.

We therefore stimulated BM-DC with increasing numbers of bacteria, in the absence or presence of LPS. Figure 2A shows the production of TNF-α, IL-6 and IL-12p70upon

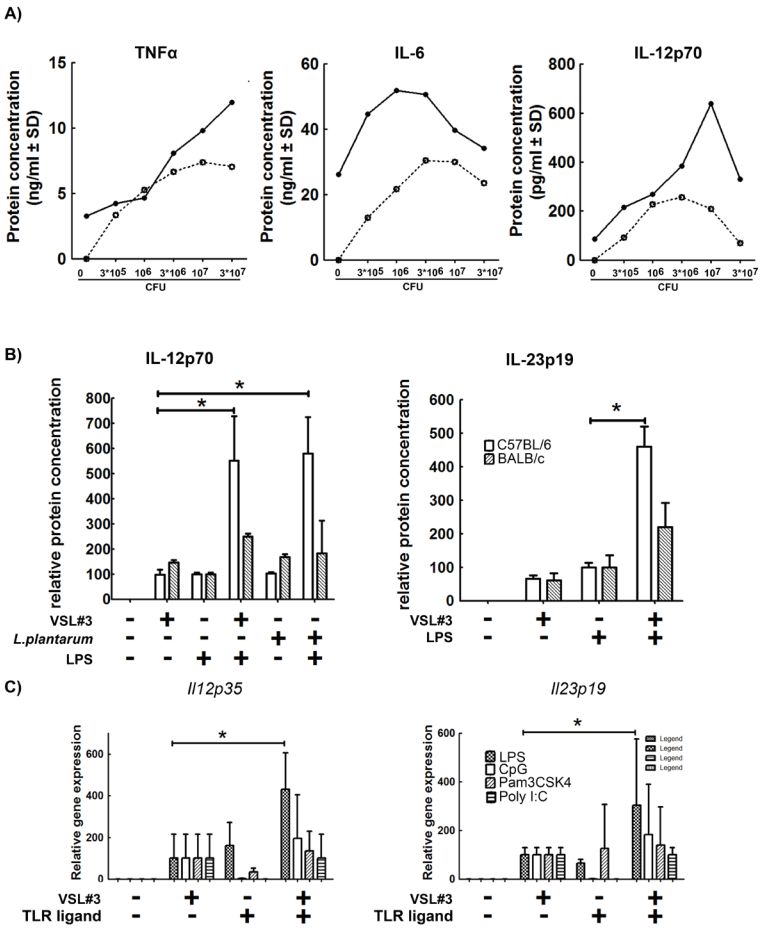

Figure 2. BM-DC from C57BL/6 and BALB/c mice differ in their response to probiotic bacteria and pure TLR agonists.

(A) Production of IL-6, TNFα, and IL-12p70 by BM-DC from C57BL/6 mice, stimulated with various VSL#3 concentrations (CFU/ml) in the absence or presence of 1 µg/ml LPS.

(B) Synergistic induction of IL-12p70 and IL-23 depends on the genetic background of the bone marrow donor mouse. IL-12p70 and IL-23 secretion by BM-DC from C57BL/6 mice (white bars) or BALB/c mice (hatched bars) stimulated with bacteria, LPS or a combination of both, is shown as a percentage of cytokine levels secreted in response to LPS alone (C57BL/6: 138 pg/ml IL-12p70 and 117 pg/ml IL-23; BALB/c: 14 pg/ml IL-12p70 and 258 pg/ml IL-23) . Results represent the mean ± SD of 3 mice per group. Mann-Whitney U test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data are representative for 2 to 3 independent experiments.

(C) Relative *Il12p35*and *Il23p19* gene expression by C57BL/6 BM-DC after stimulation with LPS (1 µg/ml), CpG (5 µg/ml), Pam_3CSK_4 (100 ng/ml) and poly I:C (50 ng/ml). Results are expressed as a percentage (mean ± SD of 3 individual mice) of mRNA expression, induced by stimulation with VSL#3 alone. Synergistic induction was only found with the combination of VSL#3 and LPS. Results were evaluated statistically using the Mann-Whitney U test; * $p<0.05$.

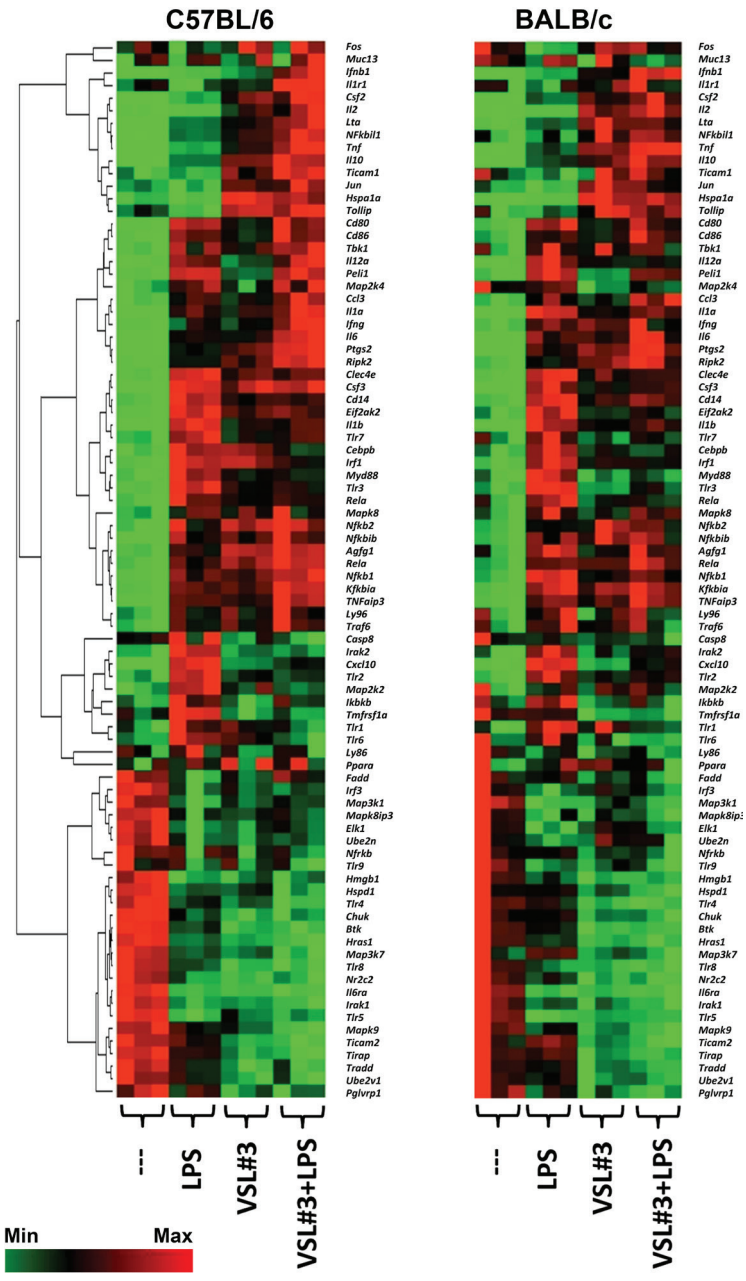

Figure 3. Differences in gene expression between BM-DC from C57BL/6 mice and BALB/c mice.

Clusterogram showing the supervised hierarchical clustering of genes related to TLR-signaling after stimulation of BM-DC with medium, LPS (1 µg/ml), VSL#3 (10^7 CFU/ml), or a combination of both. Relative expression levels for each individual gene are presented as minimum (green) and maximum (red). Columns represent gene expression profiles of BM-DC from C57BL/6 and BALB/c mice, 4 hours after stimulation (3 individual mice per strain and experimental condition).

stimulation of C57BL/6 BM-DC. All of these cytokines were stimulated by VSL#3 in a dose-dependent fashion. Simultaneously, VSL#3 strongly induced IL-10 production (supportive Figure 1). The combination ofVSL#3 and LPS resulted in an additive effect on the secretion of all of these cytokines. However, at a VSL#3:DC ratio of 10:1 a synergistic induction of IL-12p70 was observed.

Figure 2B shows that BM-DC from C57BL/6 mice showed a synergistic increase of IL-12p70 and IL-23in response to VSL#3 and LPS, whereas such an effect was not observed with BM-DC from BALB/c mice. This difference in response of BM-DC from C57BL/6 and BALB/c mice was confirmed for IL-12p70 with *L. plantarum,* one of the constituents of VSL#3 (Figure 2B). Recently, it has been shown that TRIF- and MyD88-dependent TLR ligands act in synergy to induce the release of IL-12p70 in BM-DC[16].However,VSL#3 did not show synergy with the MyD88-coupled TLR-ligands CpG and Pam_3CSK_4, or the TRIF-coupled TLR ligand poly I:Cwith regard to the induction of IL-12p70 and IL-23p19(Figure 2C). Synergy in IL-12p70 and IL-23 induction was hence specific for the combination of VSL#3 and LPS, and only found with BM-DC from C57BL/6 mice.

TLR gene array analysis of BM-DC identifies stimulating and blocking activities of VSL#3

To identify mechanisms by which probiotic bacteria may modulate the innate immune response, we analyzed mRNA expression of 84 genes implicated in TLR signaling. A time course study, revealed peak levels of mRNA encoding IL-12p35, IL-12p40, and IL-23p19 after 4 hours of stimulation with VSL#3 and LPS (data not shown). Therefore, RNA was isolated from BM-DC after 4 hours of culture with or without LPS, VSL#3, or a combination of both. Figure 3 shows the relative gene expression in BM-DC from C57BL/6 and BALB/c mice. Results of three individual mice per stimulus are each shown as a column in this heatmap. Hierarchical clustering of transcripts in C57BL/6BM-DC revealed genes that are co-regulated. In BM-DC derived from both mouse strains, LPS and VSL#3 down-regulated a cluster of genes encoding TLR, e.g.*Tlr5, Tlr4* and *Tlr8*. Moreover, both VSL#3 and LPS induced a set of pro-inflammatory gene transcripts, such as *Tnf*, *Il1a* and *Il6*, regardless of mouse strain.

In addition to the synergistic induction of IL12 and IL23(Figures 2B and 2C), the TLR signaling array revealed that the combination of VSL#3 and LPS also synergistically induced the expression of several other genes involved in innate immunity. In Figure 4, it is shown that 3 representatives of this cluster - *Lta*, *Ifng* and *Ifnb*–are synergistically induced in C57BL/6 BM-DC, but not in BALB/c BM-DC.

Importantly, a set of LPS-induced gene transcripts was suppressed by VSL#3 both in BALB/c BM-DC and in C57BL/6BM-DC.In this cluster *Myd88*, *Cxcl9* and *Cxcl10* were the most important representatives.

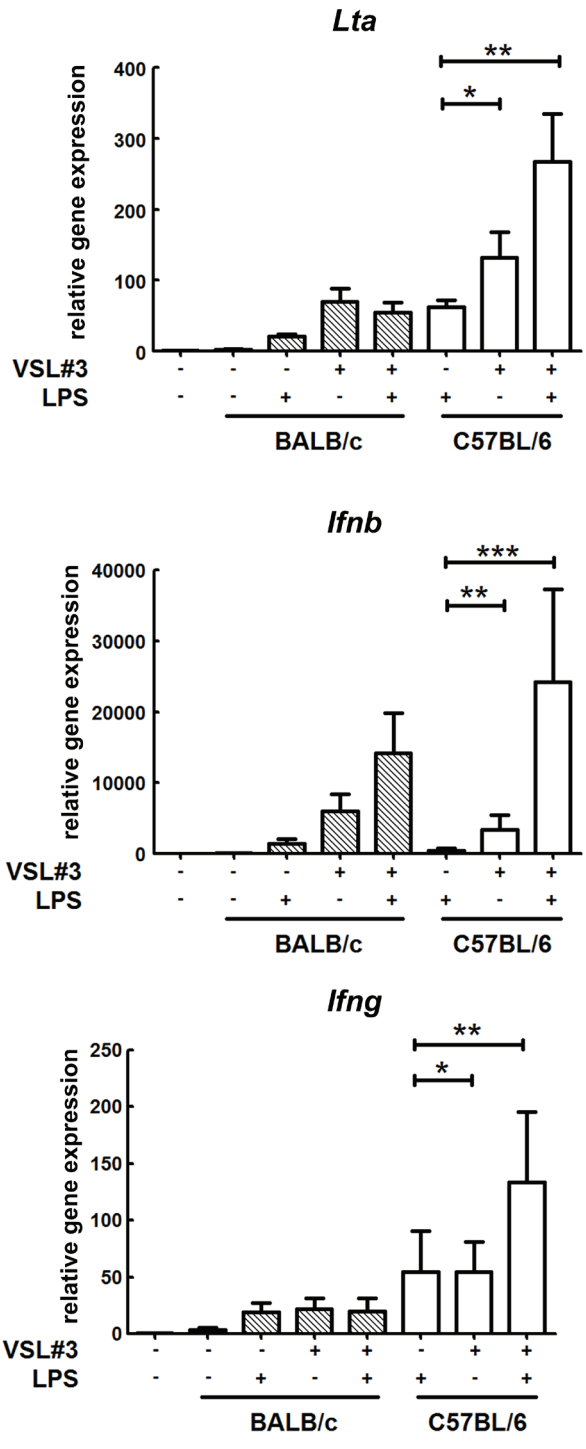

Figure 4. VSL#3 and LPS show synergistic induction of a cluster of genes in BM-DC from C57BL/6 mice, but not from BALB/c mice. The expression level (i.e. C_t values obtained from Figure 3) of *Lta* (top), *Ifnb* (middle) and *Ifng* (bottom) in BM-DC from C57BL/6 or BALB/c mice was determined after stimulation with VSL#3 (10^7 CFU/ml), LPS (1 µg/ml) or a combination of both. Bar graphs represent the fold induction compared to unstimulated C57BL/6 BM-DC. Statistical evaluation was performed with the Student's t-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

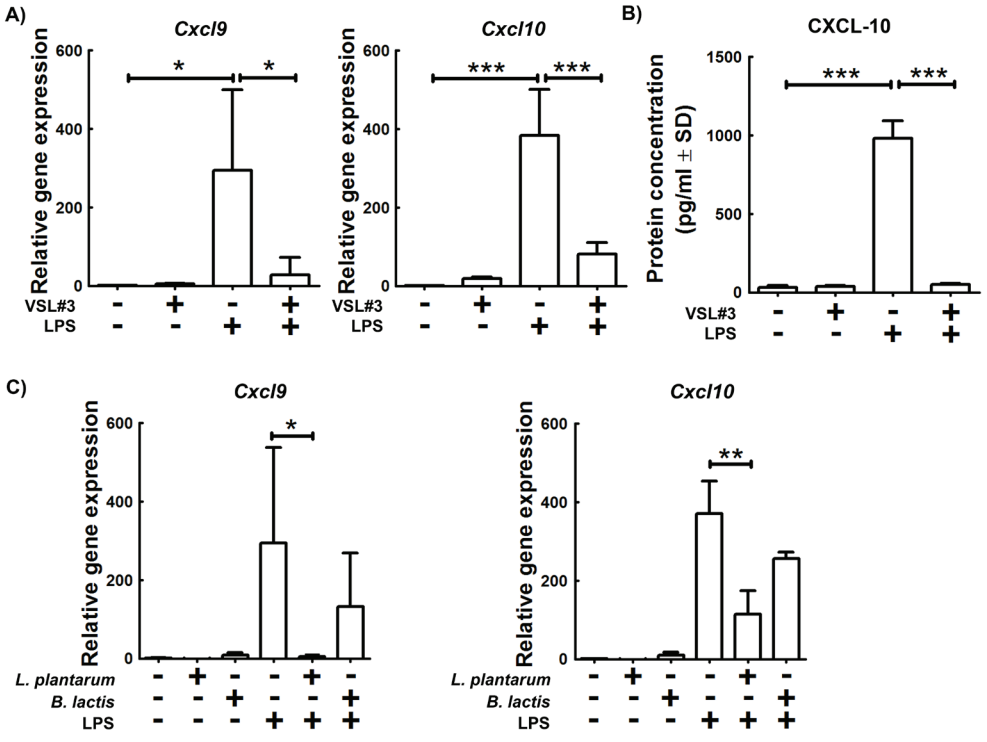

Figure 5. Suppression of LPS-induced chemokine induction by VSL#3 and *L. plantarum*.
(A) mRNA expression level for *Cxcl9* and *Cxcl10* in BM-DC from C57BL/6 mice after stimulation with VSL#3, in the absence or presence of LPS (1 µg/ml). Data are presented relative (mean ± SD of three individual mice) to unstimulated cells. For statistical evaluation the Mann-Whitney U-test was used. * p<0.05, ** p<0.01, *** p<0.001. Data are representative for 3 independent experiments.
(B) CXCL-10 production after 24 hours of stimulation was determined by multiplex assay.Mean concentrations ± SD measured in culture supernatants of BM-DC from 3 individual mice are presented. Mann-Whitney U-test; *** p<0.001.
(C) Effect of *L. plantarum* and *B. lactis* on LPS-induced expression of *Cxcl9* and *Cxcl10* in BM-DC from C57BL/6 mice. Data represent mean fold change ±SD of three individual mice, compared to unstimulated cells. Results were evaluated statistically using the Mann-Whitney U test. * p<0.05, ** p<0.01, *** p<0.001.
(D) CXCL-10 production after 24 hours of stimulation was determined by multiplex assay. Mean concentrations ± SD in culture supernatants corresponding with three individual mice are presented. Mann-Whitney U-test; ** p<0.01.

The suppressive effect of VSL#3 on LPS-induced *Cxcl9* and *Cxcl10* expression was verified by quantitative RT-PCR (Figure 5A).Down-regulation was also confirmed on the protein level for CXCL-10 (Figure 5B);levels of CXCL-9 were below the detection limit.
VSL#3 is composed of different members of two lactic acid genera, i.e. Lactobacilli and Bifidobacteria. We therefore studied effects of representative members, i.e. *L. plantarum* and *B. lactis.* Whereas *L. plantarum* suppressed LPS-induced CXCL-10 both at the mRNA and protein level and CXCL-9 at the mRNA level, *B. lactis* failed in this respect (Figure 5C).

Discussion

Probiotic bacteria and prebiotic supplements have successfully been applied in the treatment of various inflammatory disorders, including allergies and intestinal disorders [17]. However, results of clinical trials are not consistent and variation in efficacy most likely depends on numerous factors, including type of bacterial strain (single strain versus a mixture of strains), dosing regimen, delivery method and host factors, such as genetics, age, diet, and disease state [18]. Several trials have shown beneficial effects of lactobacilli, bifidobacteria and *E.coli* Nissle 1917 in the induction of remission as well as the maintenance of remission in patients with Ulcerative Colitis [19]. However, the efficacy in Crohn's disease appears less evident [20]. In the underlying study, we employed a simplified model system to evaluate the effects of VSL#3 - a complex mixture of TLR ligands - on BM-DC and identified both pro- and anti-inflammatory effects. These apparently contradictory findings may be due to the simultaneous presence of eight probiotic strains. On the other hand, similar contradictory effects were observed with *L. plantarum*, i.e. one single probiotic strain. In fact, even individual TLR ligands may induce a quick and transient production of pro-inflammatory cytokines such as IL-12, followed by the production of anti-inflammatory cytokines such as IL-10, as part of a suppressive mechanism essential to limit the extent of inflammation. The balance between (the levels) of these cytokines may differ between individuals and represents a major determinant for the severity of the subsequent inflammation process. Besides, probiotic bacteria may mediate additional mechanisms *in vivo* that control the extent of inflammation, e.g. the enhancement of intestinal barrier function, altered epithelial signaling, competition with pathogens and secretion of short chain fatty acids like acetate, propronate and butyrate, and have also effects on immune cells distinct from DC [21-23].
Although we allowed bone marrow progenitors from BALB/c versus C57BL/6 mice to differentiate *in vitro* into immature DC under controlled and identical conditions, we observed that VSL#3 and several TLR-ligands induced a more pronounced pro-inflammatory response in C57BL/6 mice , whereas BALB/c rather showed more IL-10 production. These observations are consistent with differences between the two mouse strains in terms of cellular and

humoral immune responses to different pathogens [24-26]. Our study did not show that levels of TLR gene expression could explain these differences. Moreover, we observed in BM-DC from C57BL/6 mice, but not from BALB/c mice, that VSL#3 augmented the induction of IL-12p70 and IL-23 by LPS in a synergistic fashion. Furthermore, several other genes involved in cellular immunity, were synergistically up-regulated after combined stimulation with VSL#3 and LPS. This suggests that this mixture of probiotic bacteria carries the potential to amplify inflammatory responses dependent on the genetic background of the host.

In parallel studies employing human DC, we demonstrated that LPS-induced phosphorylation of STAT-1 was inhibited by VSL#3, whereas phosphorylation of NF-κB was not influenced (Mariman et al., submitted for publication). A similar mechanism could explain the inhibition of chemokines, such as CXCL-9 and CXCL-10 by VSL#3, along with a robust induction of IL-12 and IL-23.

It should be noted that effects of VSL#3 are not representative for individual probiotic strains, as was shown by comparing *L. plantarum* and *B. lactis* in our *in vitro* studies.

Our findings may also explain that certain probiotic strains were ineffective *in vivo* or even displayed adverse effects during ongoing inflammation [27,28]. On the other hand, our extensive characterization of gene expression during the induction of TNBS-colitis in BALB/c mice [8] did not reveal that VSL#3 enhanced IL-12 p40 or IL-23p19 mRNA levels in colon tissue (even not in acute phases of the model). As mentioned above, this might be due to simultaneous induction of anti-inflammatory cytokines, or dampening of the local inflammatory response through mechanisms distinct from TLR-signaling.

Importantly, in this *in vitro* study we confirmed that probiotic bacteria may down-regulate the production of chemokines in agreement with our *in vivo* findings in the recurrent TNBS colitis model, where we also showed less recruitment of inflammatory cells and a lower disease severity[29].

Altogether, our data suggest that the net effect of probiotic bacteria *in vivo* depends on a complex of factors that vary between individuals and may depend on their genetic background and health status.

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Financial interest

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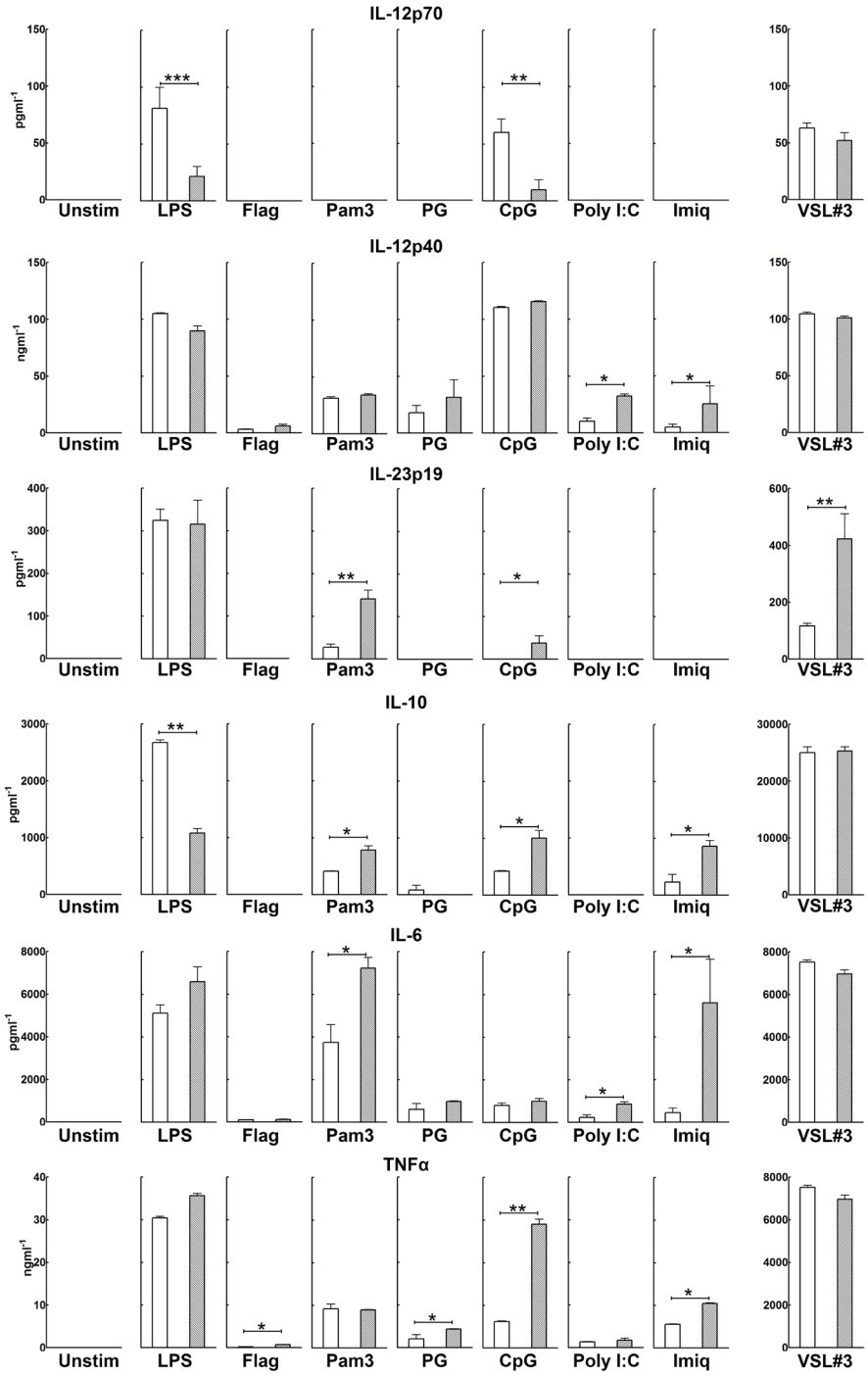
Supporting Figure 1. BM-DC from C57BL/6 and BALB/c mice respond differently to TLR-ligands.

Induction of key polarizing cytokines depends on the genetic background of the bone marrow donor mice. BM-DC were stimulated for 24 hours with 100 ng/ml Pam_3CSK_4, 50 µg/ml peptidoglycan (PG), 100 µg/ml poly I:C, 1 µg/ml LPS, 500 ng/ml Flagellin (Flag), 1 µM Imiquimod (Imiq), 5 µg/mlCpG or 10^7 CFU/ml VSL#3. Supernatants were evaluated by ELISA with regard to cytokine secretion for C57BL/6 mice (white bars) or BALB/c mice (hatched bars). Means concentrations ± SD of 3 individual mice are presented. Mann-Whitney U test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.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Chapter 6

Probiotics dampen TLR-4 mediated activation of human dendritic cells by inhibition of STAT-1 phosphorylation

Authors

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Submitted for publication

Abstract

Probiotic bacteria represent a complex of microbe-associated molecular patterns by which they are suggested to mediate health promoting effects on the host. We recently demonstrated in a mouse model of colitis that such effects may in part be due to suppression of chemokine expression in the colon. Moreover, *in vitro* studies implicated a role for probiotic bacteria in the suppression of LPS-induced chemokine production by mouse bone marrow derived dendritic cells. Herein, we studied modulation of human monocyte-derived DC by a mixture of probiotic bacteria in response to the TLR-4 agonist LPS and evaluated chemokine mRNA expression and protein secretion. This approach revealed differences in gene expression by LPS or VSL#3, a mixture of probiotic bacteria. Moreover, a set of LPS-induced chemokines was identified that was suppressed by VSL#3. *In silico* approaches identified STAT-1 as a dominant regulator of this cluster, and this was confirmed by demonstrating that phosphorylation of this transcription factor was inhibited by VSL#3.These data indicate that probiotic bacteria may contribute to the control of inflammation by selective suppression of chemokines.

Introduction

The gastrointestinal tract is continuously exposed to foreign antigens – mainly derived from the commensal microflora and/or food antigens [1]. Immune responses in the intestinal mucosa need to be tightly regulated by activating pro-inflammatory pathways for appropriate host defense against pathogenic microorganisms while remaining unresponsive to symbiotic bacteria [2,3].Under homeostasis conditions little or no inflammation occurs in the gut associated lymphoid tissue (GALT). However, genetic defects and impairment of barrier integrity may cause exaggerated immune responses driven by the microflora resulting in the development of intestinal inflammation [4].Specific lactobacilli and bifidobacteria have been shown efficient in modulating intestinal immunity in homeostasis [5]and conditions of chronic intestinal inflammation like inflammatory bowel disease [6]. Several modes of action of probiotic bacteria have been identified, including restoration of microbial homeostasis through microbe-microbe interactions, pathogen inhibition, enhancement of barrier integrity, or via direct modulation of immune responses [7].

In the gastro-intestinal tract, probiotic bacteria may be recognized by a plethora of pattern recognition receptors, including toll-like receptors, C-type lectin receptors and NOD-like receptors on epithelial cells and innate immune cells, such as dendritic cells (DC) [8,9]. Maturation of DC after contact with antigen or inflammatory stimuli is accompanied by functional and phenotypic changes. Dependent on the stimulus, DC can secrete chemokines and cytokines, thereby inducing naïve T cell proliferation and polarization towards Th1, Th2, Th17 effector cells or regulatory T cells[10].Accordingly, much attention has focused on the impact of DC priming by probiotic bacteria to modulate adaptive immune responses. Recently, we demonstrated that treatment with VSL#3 has profound effects on gene expression in the colon of mice subjected to colitis induction, with favorable effects on disease development [11]. These data were suggestive for a role of probiotic bacteria in the modulation of innate immunity, in particular chemokines. Moreover, *in vitro* studies with mouse bone marrow derived DC implicated a role for VSL#3 by blocking the ability of LPS to induce various chemokines DC (Mariman *et al,* submitted for publication).

In this study, we examined human monocyte derived DC and the modulation of cytokine and chemokine expression by VSL#3, employing dedicated PCR arrays and multiplex protein profiling. By this approach we identified a cluster of LPS-induced genes that were suppressed by VSL#3. STAT-1 was identified as a dominant regulator of this cluster, and potential target of probiotic bacteria.

Material and methods

DC cultures

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors (Sanquin, Leiden, the Netherlands) by Histopaque (Sigma Diagnostics St.Louis, MO, USA) density gradient centrifugation. Human monocytes were purified from PBMC by positive selection using anti-CD14-conjugated magnetic microbeads (StemCell Technologies, Grenoble, France). The recovered cells were 95% to 99% $CD14^+$ as determined by flow cytometry. Monocytes were cultured at 2×10^6/ml in RPMI1640 supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 10µMultraglutamin and 5% (v/v) FBS (Lonza, Verviers, Belgium). In order to stimulate their differentiation into DC, cells were cultured in the presence of GM-CSF (50 ng/ml) and IL-4 (40 ng/ml). After 5 days of culture, immature DC were washed and seeded at 10^6/well in 24-well plates. Stimuli – all in culture medium – were added to these DC and after 48 hours of culture supernatants were harvested for cytokine and chemokine profiling. For RNA profiling, RNA was isolated after 4 hours of culture.VSL#3, a mixture containing 8 different lactic acid bacteria, was purchased from Ferring Pharmaceuticals (Berkshire, UK). Freshly reconstituted bacteria from lyophilized stocks were added to DC cultures at a bacteria – DC ratio of 10 to 1. Ultrapure LPS*E. coli K12* (final concentration 1µg/ml) was purchased from Invivogen (San Diego, California, USA).

RNA isolation and transcription profiling using PCR array

Total RNA was isolated from 10^6 cells using a RNAEasy Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. 500 ng RNA was used for single stranded cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA USA) and incubated for 60 min at 37 ^oC and 5 minutes at 95 ^oC.cDNA was used for PCR-array analysis by using the RT^2-ProfilerPCR Array (human cytokine & chemokine array) from SuperArray Bioscience (Frederick, MD, USA). The PCR array combines the quantitative performance of SYBR Green-based real-time PCR with the multiple gene profiling capabilities of microarray. 96-well plates containing gene-specific primer sets for 84 chemokines and cytokines, 5 housekeeping genes, and 2 negative controls were used. For each experimental condition, cDNA from 6 individual donors was analyzed. Gene expression was normalized to internal controls (housekeeping genes) to determine the fold change in gene expression between test and control samples by $\Delta\Delta C_t$ (SuperArray Bioscience).

Chemokine profiling

Multiplex analysis was performed on the supernatants of DC for profiling of secreted chemokines (Invitrogen, human 10-plex)according to the manufacturer's instructions. The beads were read on a LiquiChip 200, (Qiagen, Hombrechtikon, Switzerland), and data

were analyzed by the five parameter curve fitting in Luminex100 IS Software. Chemokine concentrations are presented as pg/ml.

NF-κB and STAT-1 phosphorylation assay

STAT-1 transcription factor activity was quantitatively detected by TransAM STAT family transcription factor assay (Active Motif, Carlsbad, CA) according to the manufacturer′s recommendations. In short, cell nuclei were prepared from DC using a nuclear extraction kit (Active Motif). Nuclear lysates (derived from $15*10^6$ cells) were incubated in 96-well dishes containing immobilized oligonucleotides containing a STAT consensus DNA-binding site (5′-TTCCCGGAA-3′). The specific primary antibody and the secondary antibody conjugated to horseradish peroxidase were incubated with the nuclear extracts following the vendor's manual. The colorimetric reading (at 450 nm) was determined on a VERSAmaxmicroplate. Nuclear extracts from COS-7 cells (treated with IFN-γ) were included as positive controls for STAT1α. Phosphorylation assay for NF-κBp65 was performed as described for STAT-1, with slight modifications. Instead of TMB substrate, a chemiluminescent substrate was used to quantify phosphorylation of this transcription factor.

Results& Discussion

LPS and VSL#3 induce distinct cytokine/chemokine expression profiles in human DC

We previously identified the modulation of chemokines as a major mechanism in the protective effect of probiotic bacteria in colitis[11]. These effects could be partly mimicked *in vitro* employing mouse bone marrow derived DC. Consequently, we wished to study if and how probiotic bacteria would modulate inflammatory responses in human DC. Therefore, we evaluated modulation of TLR-4 induced signaling pathways in human DC by probiotic bacteria. DC from 6 individual donors were generated by culturing $CD14^+$ monocytes in the presence of GM-CSF and IL-4. After 5 days of culture cells were washed and stimulated with LPS, the probiotic mixture VSL#3 or a combination of both.

Consistent with previous publications [12-14],the mixture of lactic acid bacteria as well as LPS were able to induce DC maturation, evident from the enhanced expression of HLA-DR, CD86, and CD83 (data not shown).

To assess the expression of chemokine and cytokines, RNA was isolated from DC left unstimulated or stimulated with LPS, VSL#3, or a combination of both, and subsequently analyzed with the use of a dedicated PCR gene array. A complete list of all measured transcripts and their fold induction relative to unstimulated DC is depicted in supportive Table 1. Transcripts of a large number of pro-inflammatory mediators were induced compared

Supportive Table 1 mRNA expression levels of 84 chemokines and cytokines after 4 hours of stimulation with LPS, VSL#3 or a combination of both, as determined by qPCR arrays. Data for each gene were normalized to a panel of housekeeping transcripts and compared to unstimulated DC. Results represent the mean of 6 individual donors.

	Up–regulation (as compared to medium control) Fold change					
Chemokine/Cytokine	**LPS**		**VSL#3**		**VSL#3 + LPS**	
ADIPOQ	1.4		1.2		1.4	
BMP2	3.5		2.4		8.4	
BMP4	1.1		1.9	*	1.7	*
BMP6	3.6		14.4	**	52.3	**
BMP7	-1.0		1.4		1.4	
C5	-1.4		-1.8		-1.1	
CCL1	762.7	**	210.8	*	234.3	*
CCL11	1.6		1.8		2.7	**
CCL13	2.0		-1.3		-1.1	
CCL17	2.9	**	1.9		2.3	*
CCL18	1.0		-1.0		1.1	
CCL19	45.7		4.4	**	18.0	
CCL2	45.6	**	19.1	*	16.2	**
CCL20	954.5	***	755.3	*	1657.4	***
CCL21	1.3		-1.1		1.1	
CCL22	5.6	**	8.1	**	8.8	**
CCL24	1.2		1.4		1.0	
CCL3	47.7	***	105.2	**	159.5	***
CCL5	412.7	**	127.9	*	476.7	**
CCL7	6.8	*	4.4		3.1	
CCL8	145.8	**	7.8		13.2	***
CD40LG	-2.1		-2.7	*	-1.3	
CNTF	-1.4	*	-1.4		-1.3	
CSF1	14.9	**	23.0	**	26.9	**
CSF2	182.0	***	687.2	*	2049.1	***
CSF3	50.9		51.1		233.0	
CX3CL1	4.5	*	3.2		2.6	
CXCL1	589.9	**	403.5		1060.3	**
CXCL10	8118.3	***	217.6	**	1488.8	***
CXCL11	30832.8	**	294.6	***	7989.2	**
CXCL12	1.8		1.3		1.7	
CXCL13	1.4		-2.7	*	-1.7	
CXCL16	1.4	*	2.0	**	2.2	***
CXCL2	153.5	***	452.8		1088.3	**
CXCL5	4.1	*	7.9	*	6.5	*
CXCL9	4136.7	*	42.5	**	602.7	**
FASLG	7.1		4.8	**	10.2	*
GPI	-1.4		-1.6	*	-1.5	*
IFNA2	-1.0		1.1		1.5	
IFNG	41.5	*	36.2		94.0	*

	Up–regulation (as compared to medium control) Fold change					
Chemokine/Cytokine	**LPS**		**VSL#3**		**VSL#3 + LPS**	
IL10	13.8		20.2		69.6	
IL11	1.8	*	2.3		3.7	*
IL12A	73.4		1591.1	*	3385.0	*
IL12B	1817.4	*	9130.1	*	5194.7	**
IL13	3.5	*	4.9	*	12.0	***
IL15	14.6	*	2.7	*	9.7	***
IL16	-10.6	***	-6.2	***	-8.2	***
IL17A	-1.0		1.4		1.3	
IL17F	2.0	*	1.5		2.4	**
IL18	-2.7	*	1.7	*	-1.5	
IL1A	691.8	*	925.2	*	1771.6	***
IL1B	567.1	*	895.1	*	1560.5	**
IL1RN	8.4	*	19.8	***	25.2	***
IL2	2.1	*	3.6	*	3.5	*
IL21	1.3		-1.0		2.0	**
IL22	1.8		1.1		2.3	
IL23A	34.1	*	104.4	***	300.6	*
IL24	-1.2		1.3		1.3	
IL27	127.6	*	27.5		144.4	
IL3	-1.0		-1.2		1.4	
IL4	1.4		1.2		1.7	
IL5	1.1		-1.4		1.4	
IL6	2184.1	**	1657.6		3885.9	
IL7	142.2	*	86.0		199.7	*
IL8	323.6	***	632.7	*	1090.3	**
IL9	-1.2		4.7		2.2	
LIF	33.2	***	246.7		284.0	
LTA	57.2	***	168.4	***	157.7	***
LTB	1.0		1.1		1.2	
MIF	1.1		1.1		1.2	
MSTN	-1.4		-1.9	*	-1.3	
NODAL	-2.4	*	-2.3	*	-2.5	*
OSM	4.3	***	7.4	**	13.2	*
PPBP	-1.4		-1.2		-1.2	
SPP1	1.2		6.5	***	4.6	**
TGFB2	-3.1	***	-2.5	***	-1.6	*
THPO	1.1		-1.1		-1.1	
TNF	50.4	***	206.5	*	248.0	***
TNFRSF11B	9.6	*	18.0	*	32.3	***
TNFSF10	137.5	***	6.9	*	18.2	***
TNFSF11	1.3		1.3		1.4	
TNFSF13B	1.2		-3.6	**	-1.9	
VEGFA	2.8		7.7	*	12.5	*
XCL1	-1.5		1.5		1.9	

Results were statistically evaluated by Student's t-test, comparing the replicate 2^(- delta Ct) values for each gene in the control group and treatment groups.
* p<0.05, ** p<0.01, and *** p<.0001.

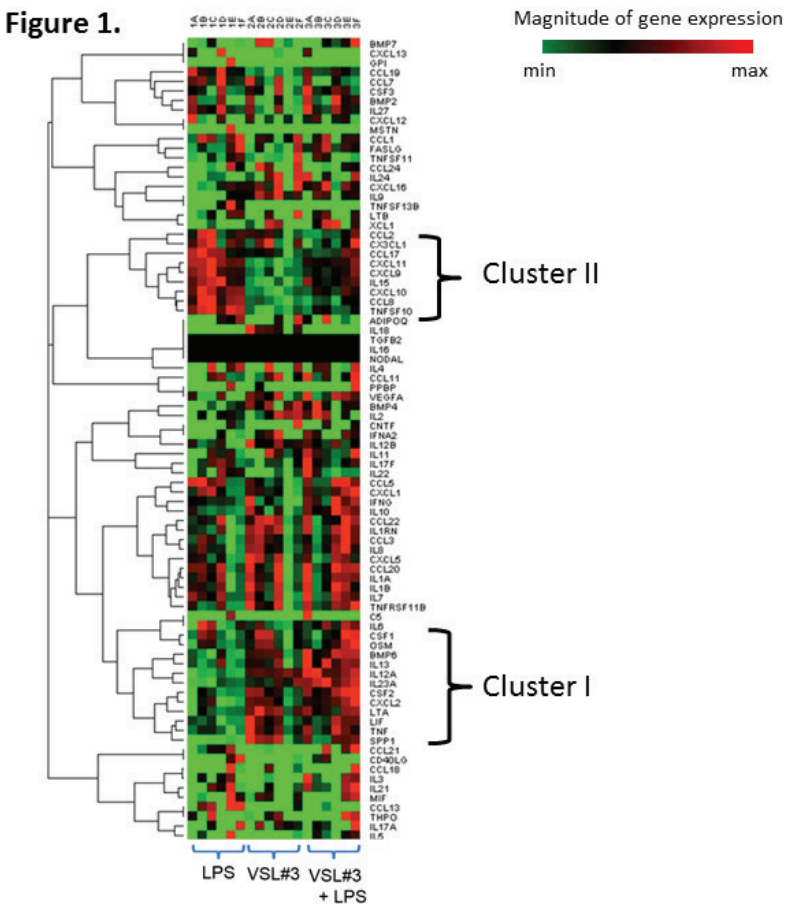

Figure 1.
Chemokine and cytokine expression by stimulated DC.
Clusterogram showing the supervised hierarchical clustering of relative expression levels for all samples and all genes included in the real-time PCR arrays. Data for each gene were normalized to a panel of housekeeping transcripts and compared to unstimulated DC. Relative expression levels for each individual gene are presented as minimum (green) and maximum (red). Rows represent gene expression profiles of individual samples, 4 hours after stimulation (6 donors for each experimental condition).

to immature DC, irrespective of the stimulus. However, the magnitude of induction or inhibition was gene-and stimulus-specific. Therefore, hierarchical clustering was applied to identify clusters of co-regulated genes. A heatmap showing the clustering of the expression patterns of 6 individual donors is depicted in Figure 1. In this heatmap, minimum (green) and maximal (red) fold inductions as compared to unstimulated DC are shown for each gene. Two major cluster could be identified as indicated in Figure 1.Cluster I comprise genes that are moderately induced by LPS, compared to the mixture of bacteria. These include *Il1α,*

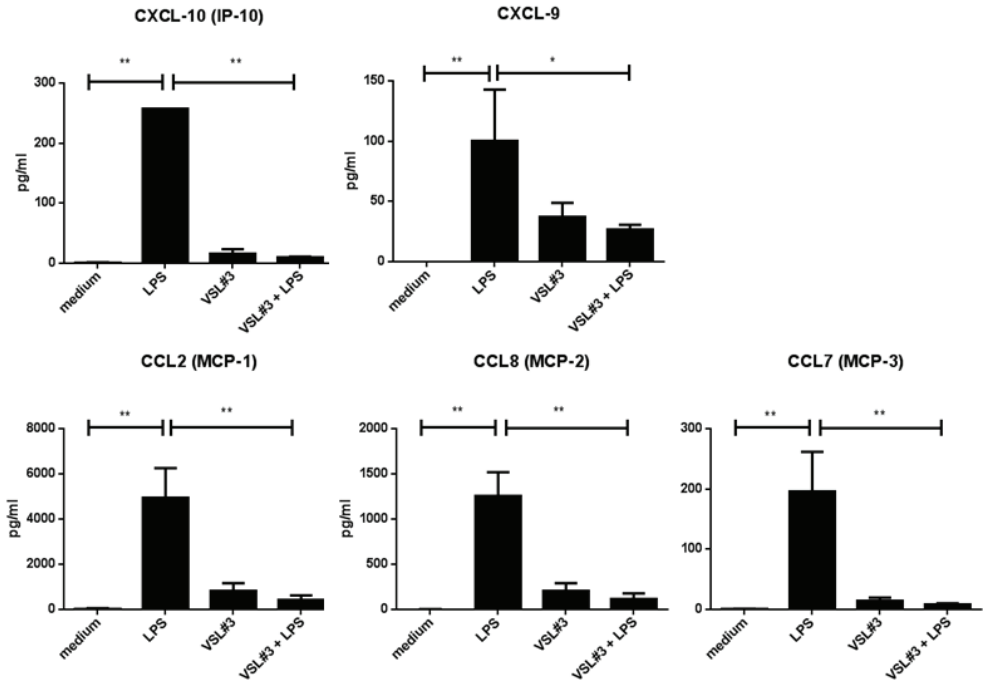

Figure 2.
LPS-induced chemokine production of human DC is suppressed by VSL#3
A set of genes representing cluster II was confirmed at the protein level. Supernatants were harvested from replicate DC cultures after 48 hours and performed in parallel with the cultures used for mRNA profiling. Levels of various cytokines and chemokines were determined by multiplex bead array technology. Results are expressed as pg/ml ± SD.
Rank-Wilcoxon paired test: * p<0.05, ** p<0.01, *** p<.0001

*Il1β,Ccl3, Csf2, Cxcl2, Il12a, Il12b.*Moreover, the co-incubation of human DC with VSL#3 and LPS synergistically induced *IL-23A* and *IL12A* transcripts. Together with *IL-12B*, these subunits form IL-23 and IL-12p70, respectively. Secretion of these cytokines by DC may polarize naïve T-cells into T_h17 and $_{Th1}$ cells. Robust responses with respect to IL-12, but also IL-10 are in line with previous reports in human and mouse DC [15,16], and are related to high bacterial doses.

Interestingly, cluster II comprises LPS-induced genes that were suppressed by VSL#3. Cluster II comprised*Tnfsr10* and a number of chemokines, including *Cxcl-9, Cxcl-10, Cxcl-11, Ccl1, Ccl2, Ccl8*, and *Ccl19*. Supernatants of replicate cultures harvested after 48 hours were subjected to multiplex analysis and this confirmed diminished protein levels of CXCL-9, CXCL-10, CCL-2 and CCL-8 as shown in Figure 2. It is known that LPS can bind to TLR4 and thereby inducing both pro – and anti-inflammatory effects [17]. At the level of the plasma membrane, LPS activated TLR-4 binds to sorting adaptor (TIRAP), which then recruits the signaling adaptor myD88 to trigger production of pro-inflammatory mediators. However,

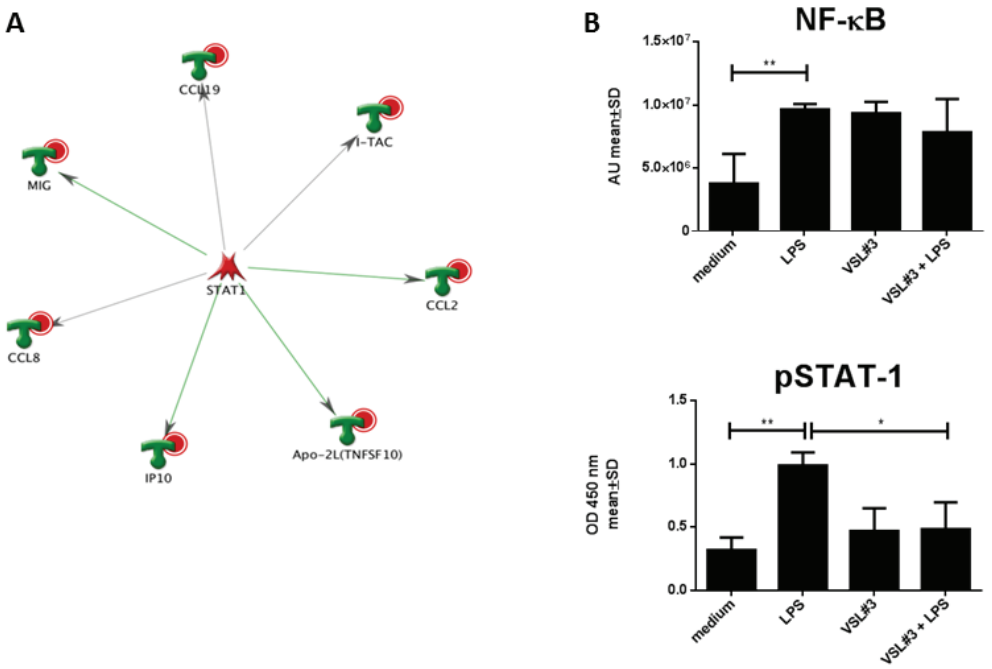

Figure 3.
VSL#3 inhibits LPS-induced phosphorylated STAT-1
(A) LPS-induced genes, significantly suppressed by VSL#3, were analyzed by using Metacore Network analysis (Transcription factor). STAT-1 was predicted to be a key driver of this cluster.
(B) DC were stimulated for 3 hours, and STAT-1 and NF-κB p65 phosphorylation was quantified in nuclear extracts using the Trans-am assay. Absorbance values (mean ± SD) of 5 individual donors are shown. Rank-Wilcoxon paired test: * $p<0.05$, ** $p<0.01$.

activated TLR-4 in the endosomes engages the adaptor molecules TIRAP and TRAF to induce IFN-β and the anti-inflammatory IL-10 [18]. It has been reported that involvement of these two signaling pathways in LPS induced chemokine expression varies considerably depending on the kind of chemokine [19]. CCL-2, CCL-7, CCL-8, CXCL-2, CXCL-3, and CXCL-9 largely rely on myD88, whereas CCL-5, CXCL-10 and CXCL-13 are regulated by myD88 independent pathways. Since in this study VSL#3 inhibited both expression of myD88-dependent (CXCL9 and CCL8) and myD88-independent chemokines (CXCL10), it is tempting to conclude that VSL#3 does not interfere at the level of myD88-or TRIF-coupled TLR-signaling.

The observed effect on the modulation of LPS-induced chemokines might be partly beyond the level of transcriptional regulation. Recently, it was demonstrated that *L. plantarum* mediates degradation of CXCL-10 by the production of lactocepins [20]; these proteinases are derived from lactococci and lactobaccili and have substrate specificity for a class of chemokines, including CXCL-9 and CXCL-10, but not CCL5[21].

However, our study shows that probiotic bacteria also selectively suppress expression of genes encoding these chemokines.

VSL#3 inhibits LPS-induced phosphorylation of STAT-1

Since only a distinct subset of LPS-induced genes was suppressed by VSL#3, we aimed to identity a common regulator for this cluster. *In silico* analysis using MetaCore software was used to identify transcription factors regulating genes in this cluster. Compared to the reference list of all 84 genes measured on the array, LPS-induced genes suppressed by VSL#3 were highly enriched for the transcription factor STAT-1 (z-score 173, p-value $1.3*10^{-26}$) as depicted in Figure 3A. To validate the role of STAT-1 in the suppression of LPS-induced gene expression found by out bioinformatics approach, an additional experiment was performed to measure phosphorylated STAT-1 in the nucleus upon stimulation. Therefore, DC were stimulated for 45 and 180 minutes before the isolation of nuclear proteins. Nuclear extract from 10^7 DC was analyzed by TRANSam™. This ELISA-based assay allows detection and quantification of DNA-binding activity of transcription factors, in this case pSTAT-1α and pNF-κBp65. Irrespective of the stimulus, NF-κB was already activated after 45 minutes of incubation (data not shown). In contrast, 3 hour after stimulation, pSTAT-1 levels were increased in nuclear extracts of LPS-stimulated DC, but not of VSL#3 stimulated cells (Figure3B). In line with the *in silico* prediction, migration of STAT-1 to the nucleus upon phosphorylation was blocked in the presence of VSL#3. Further studies are needed to elucidate how phosphorylation of this specific transcription factor is regulated by probiotic bacteria, although induction of SOCS1 might play a role in this respect[22]. Phosphorylation of NF-κB was not inhibited by VSL#3, which explains the robust induction of IL-12p70. This suggests that VSL#3 can reduce production of specific chemo-attractants *in vitro* by selective inhibiting of STAT-1 induced transcription regulation.

STAT-1 deficiency reduced the intensity of spontaneous and chemical induced colitis [23,24]. Furthermore, a recent report showed that selective regulation of STAT-1 signaling in T-cells by fusaruside resulted in reduced production of pro-inflammatory cytokines and improved TNBS induced colitis [25]. Therefore, our previous studies showing that probiotic bacteria have a favorable effect on the development of colitis may be explained by modulation of transcriptional regulation of chemokines and cytokines.

A better understanding of how MAMPs present on probiotic bacteria modulate intracellular signaling induced by pathogenic bacteria may be helpful to identify mechanisms by which probiotic bacteria contribute to the control of inflammation. In addition, our approach with emphasis on chemokine expression by DC may help to select probiotic strains with distinct properties tailored to suppress inflammation in autoimmune or allergic disorders.

Conclusion

We showed that probiotic bacteria interfere with STAT-1 phosphorylation in human DC in conjunction with the modulation of chemokine expression and secretion. This finding may contribute to a better understanding of the mechanisms by which probiotic bacteria exert anti-inflammatory effects.

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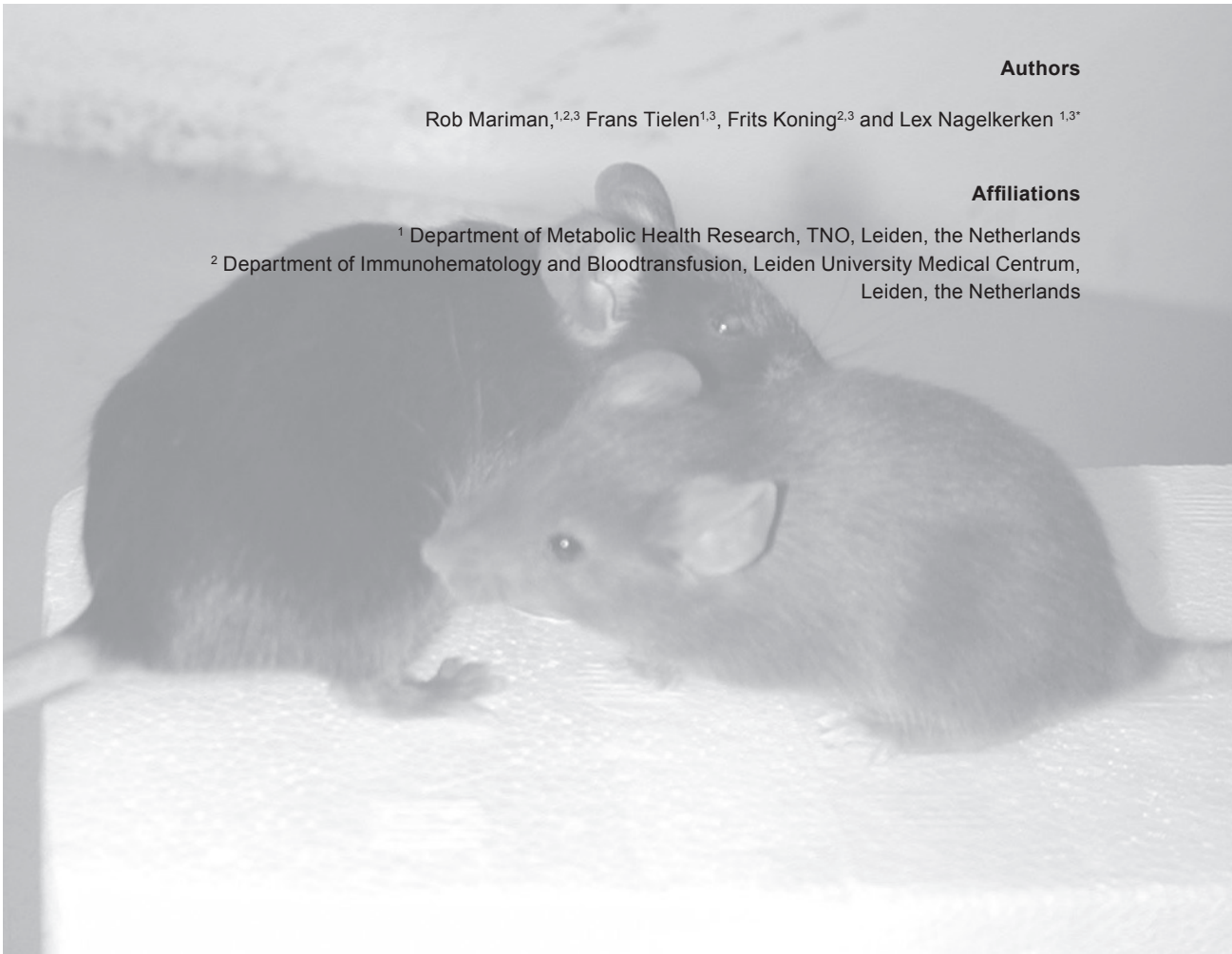
Chapter 7

Genome wide gene expression profiling reveals host-specific responses in the intestines of healthy mice in response to probiotic bacteria



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Abstract

Probiotic bacteria may render mice resistant to the development of various inflammatory and infectious diseases. For a better understanding of the underlying mechanisms by which probiotic bacteria may influence intestinal immune homeostasis, the effect of long- and short-term treatment with a mixture of 8 probiotic bacteria (i.e. VSL#3) was evaluated in non-inflammatory conditions. To this end, we studied the effect of VSL#3 in BALB/c and C57BL/6 mice, as mouse strains with dominant humoral or cellular immunity, respectively. VSL#3 administration resulted in an increase of B cells and a decrease of CD4+ T cells in the Peyer's patches (PP) and mesenteric lymph nodes (MLN) of both mouse strains. However, genome wide gene expression profiling using micro-arrays revealed that oral administration of VSL#3 to BALB/c and C57BL/6 mice was associated with host-specific modulation of gene expression in colon and small intestine. Whereas VSL#3 treatment resulted in down-regulation of *Il13* and *Epx,* and up-regulation of *Il12rb1, Ccr5, Cxcr3 and Cxcl10* in BALB/c mice, such effects were not observed in C57BL/6 mice. In BALB/c mice, a 2-fold increase in $CD103^+$ $CD11c^+$ dendritic cells was found both in PP and in MLN, 18 hours after the first treatment with VSL#3. Possibly as a consequence of this modulation of the innate immune response, prolonged treatment with VSL#3 was associated with increased numbers of Th17 cells and $Foxp3^+$ regulatory T cells in the MLN of these mice.

In conclusion, these experiments in healthy mice show that probiotic bacteria may alter the immunological phenotype of the host; the nature of these effects is dependent on mouse strain.

Introduction

Directly after birth, the (human) gastro-intestinal tract is colonized by a complex community of micro-organisms termed the “microbiota” (1). The community structure of this microbiota directly contributes to a variety of physiological and metabolic processes that are important to host function (2, 3). Moreover, interactions between microbes and the host are of critical importance in orchestrating the (mucosal) immune system (4). For example, the absence of intestinal bacteria in germ-free mice results in defects in the development of gut associated lymphoid tissue (5) reflected by reduced IgA production by plasma cells (6), fewer Peyer’s Patches (PP) and mesenteric lymph nodes (MLN) as well as impaired development of isolated lymphoid follicles (7). Likewise, the induction of either regulatory or effector T cell responses in the mucosa depends on the bacterial species present (8). For example, segmented filamentous bacteria induce effector Th17 responses, which provide protection against gut pathogens (9, 10). In addition, certain *Clostridium* species and *Bacteroides fragilis* favor the induction of $Foxp3^+$ regulatory T cell (T reg) responses (11). These T reg cells can prevent systemic and tissue-specific autoimmunity and inflammatory lesions at mucosal interfaces and are thereby essential for the maintenance of immune homeostasis. In turn, the intestinal immune system shapes the composition of the gut microbiota (12-14). In view of the interplay between the host and the intestinal microbiota, also probiotic bacteria may have favorable effects and contribute to intestinal immune homeostasis (15) (16). Beneficial effects of probiotic bacteria have been observed in patients with ulcerative colitis in remission and in patients with atopic disease (17, 18). Several mechanisms of action have been proposed, including modulation of innate and adaptive immune responses, effects on the composition of the gut microbiota, strengthening of the mucosal barrier and the prevention of microbiological translocation (19, 20). These mechanistic studies mostly rely on *in vitro* assays or animal models for inflammation (21-23). However, immunomodulation by probiotic bacteria in a simplified *in vitro* system or in an established inflammation model *in vivo* does not reflect effects in conditions of homeostasis. Only few studies have addressed the effects of probiotic bacteria in healthy subjects (24-27) and substantial variability in the response of human subjects to probiotic intervention were found (28, 29).

We previously demonstrated that prophylactic treatment with probiotic bacteria renders BALB/c mice largely resistant to the induction of colitis (30). Therefore, probiotic bacteria may represent important nutritional ingredients that contribute to intestinal immune homeostasis and protection against inflammatory disease. To gain insight into the underlying mechanism of protection, we evaluated the effect of a mixture of probiotic bacteria in healthy mice. To account for the variation among individuals, we studied this probiotic mixture not only BALB/c mice but also in C57BL/6 mice, because these mice have an established bias for Th2 and Th1 type immune responses, respectively. In this study, we show by transcriptome profiling,

in conjunction with immune cell characterization, that probiotic bacteria have differential effects on immunological processes, dependent on mouse strain.

Material and methods

Mice

9-week old C57BL/6 mice (Charles River, Maastricht, Netherlands) and BALB/c mice (Janvier, St. Berthevin, France) were used in this study. Mice were conventionally housed with free access to standard mouse chow (SSNIFF R/M-H, BioServices B.V., Uden, The Netherlands) and acidified tap water. Animal experiments were approved by the Institutional Animal Welfare Committee of The Netherlands Organization for Applied Scientific Research (TNO), approval number DEC2981 and DEC3288, in compliance with European Community regulations regarding the use of laboratory animals.

Study design

VSL#3 (Ferring Pharmaceuticals, Berkshire, UK) was purchased as a commercially available probiotic mixture containing freeze-dried bacteria (*Bifidobacterium longum, B. breve, B. infantis, Lactobacillus acidophilus, L. plantarum, L. casei, L. bulgaricus, and Streptococcus thermophilus).* To study the effect of long-term probiotic administration, mice were gavaged with PBS or 3 x 10^8 CFU VSL#3 suspended in 200 µl PBS, 3 times a week, for a period of 4 weeks. In a follow-up study, also short-term administration of probiotics was examined by sacrificing mice 18 hours after one single oral dose of VSL#3.

Flow cytometry analysis

Spleen, MLN and PP were isolated and passed through a 70-µm nylon cell strainer (BD Biosciences, San Jose, CA, USA) to prepare single-cell suspensions. Cells were stained with different rat-anti-mouse antibodies obtained from Biolegend/BD Biosciences: CD3–FITC (145-2c11), CD4–PerCp (DM4-5), CD8–PerCp-Cy7 (53-6.7), CD25-APC (PC61.5), CD45-PerCp-Cy5 (30-F11), CD19–V450 (ID3), IFN-γ–FITC (XMG1.2), IL-17–PE (TC11-18H10), CD11c–Pe-Cy7 (N418), CD103–PB (2E7) and F4/80–APC(BM8). Where needed, isotype-matched controls were included.

For intracellular cytokine staining, cells were cultured in RPMI-1640, supplemented with 10% FCS, and stimulated with 40 nM phorbol-12-myristate-13-acetate (PMA) and 2 nM calcium ionomycin (Sigma Aldrich). Brefeldin A (3 µM) was added to allow for intracellular cytokine accumulation.. Cells were stimulated for four hours at 37°C. Fixation and permeabilization reagents (Caltag Laboratories, Burlingame, CA, USA) were used for intracellular staining according to the instructions of the manufacturer. In short, 1×10^6 cells were incubated for

30 minutes with a cocktail of primary antibodies and 1% normal mouse serum to prevent non-specific antibody binding. Subsequently, cells were fixated, washed and incubated (30 minutes) with permeabilization buffer containing the intracellular cytokine antibody cocktail.. Unstimulated cell suspensions were used for regulatory T cell staining. Intranuclear Foxp3 staining was performed using eBioscience T reg staining kit using PE-conjugated Foxp3 (FJK-16s). Flowcytometric analyses were performed on a fluorescence-activated cell sorter (FACSCanto II, BD Biosciences) and results were analyzed with FACSDiva Software 6.1.2.

Transcriptome Analysis

Total RNA was isolated from frozen intestinal tissue using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. RNA was treated with DNAse and purified using Nucleospin RNAII Total RNA Isolation kit (Macherey-Nagel, Düren, Germany), according to the instructions of the manufacturer.

The quality control of RNA samples, RNA labeling and hybridization were performed at ServiceXS (Leiden, The Netherlands). The RNA concentration was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A). The RNA quality and integrity was determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). The RNA integrity numbers (RIN) of all RNA samples had values above 8.7. Biotinylated cRNA was prepared using the IlluminaTotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's specifications starting with 500 ng total RNA. Per sample, 750 ng cRNA was used to hybridize to the Sentrix MouseRef-8 BeadChips (Illumina, Inc., San Diego, CA, U.S.A.). Each BeadChip contains eight arrays and each of the arrays harbors 25,697 probes. Hybridization and washing were performed according to the Illumina standard assay procedure. Scanning was performed on the IlluminaScanner (Illumina, Inc., San Diego, CA, U.S.A.). Image analysis and extraction of raw and background subtracted expression data were performed with IlluminaBeadstudio v3 Gene Expression software using default settings. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (31) and are accessible through GEO Series accession number GSEXXXX (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSEXXXX). GeneSpring GX 11.0 was used for quantile normalization of the probe-level, background subtracted expression values. After the normalization, unexpressed probes were removed from the further analyses. All expression values below 5 (2.322 on log2 scale) were floored to 5. Differentially expressed probes were identified using the limma package of the R/Bioconductor project, applying linear models and moderated t-statistics that implement empirical Bayes regularization of standard errors (32). The statistical analyses were performed through The Remote Analysis Computation for gene Expression data (RACE) suite at http://race.unil.ch (33). p-bayes values < 0.05 was used as threshold for significance of the differential expression.

Results

Probiotic bacteria modulate gene expression in the intestinal mucosa in conditions of homeostasis

The effects of treatment with a mixture of probiotic bacteria on gene expression in colon and small intestine was evaluated in BALB/c and C57BL/6 mice. We first analyzed differences between both mouse strains with respect to gene expression in the small intestine and colon. Figure 1A shows for individual untreated mice an unbiased analysis approach using all 25,697 genes on the array for principal component analysis (PCA). This PCA-plot reveals two distinct clusters of colon and small intestine related genes (component 1), whereas component 2 reveals differences in gene expression between the two mouse strains. We next identified differentially expressed genes (fold change >1.5 and p-bayes< 0.05) between BALB/c and C57BL/6 mice for both tissues. A complete list of all differentially expressed genes can be found in Supportive Table 1. Metacore analysis revealed that overlapping genes were predominantly related to the immune response, and include MHC haplotype variation. Moreover the expression of a variety of antimicrobial peptides differed between the two mouse strains, especially in the small intestine. These antimicrobial proteins include lactoperoxidase, lysozyme and a set α-defensins. We also analyzed the expression of key regulators involved in the differentiation of CD4+ T cells. As shown in Figure 1B, the Th2 regulator GATA3 was increased in the small intestine of BALB/c mice as compared to C57BL/6 mice. In contrast, RORγ (key regulator of Th17 cells) was preferentially expressed increased in colon and small intestine of C57BL/6 mice. In view of these differences, immune modulating effects of probiotic bacteria might be different in these mice. We therefore determined the effect of VSL#3 on gene expression in both mouse strains. To this end, wild-type BALB/c and C57BL/6 mice were treated three times a week for a period of 4 weeks, by oral administration of $3x10^8$ colony forming units of VSL#3 or vehicle. Probiotic supplementation did not affect the development of body weight (data not shown). The numbers of differentially expressed genes (fold change >1.5, p-Bayes < 0.05) between VSL#3- and vehicle-treated mice are indicated in Table 1. A complete list of differentially expressed genes is provided in Supportive Table 2. To gain insight into the processes that are associated with these differently expressed genes after administration of VSL#3 to mice, significantly overrepresented Pathway Maps were created with Metacore™ Software. This unbiased approach was applied to map the differentially expressed genes into clusters with common biological functions. Table 2 shows all significant pathway maps, including the number of genes with the according p-values. This table shows profound effects of VSL#3 on genes related to the immune response. To visualize expression patterns of these immune-related genes, a heat map was constructed including all of these modulated genes in the small intestine and colon (Figures 2A and 2B, respectively). For the small intestine of BALB/c mice, these genes were further subdivided

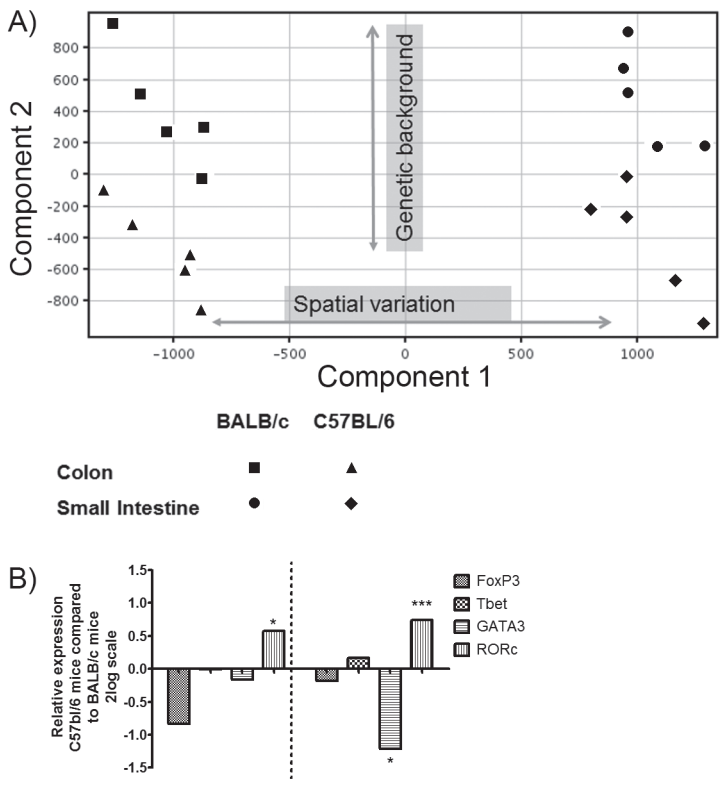
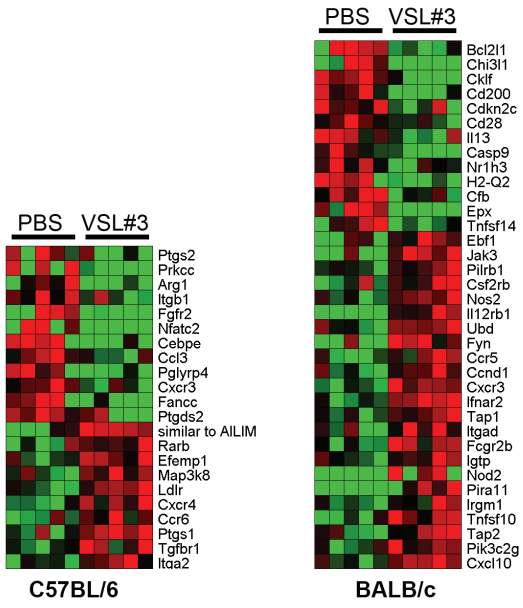


Figure 1. Gene expression profiles in the intestine of BALB/c and C57BL/6 mice
A. Two-dimensional visualization of principal component analysis (PCA) constructed with all 27,000 genes on the array. Each dot represents the expression profile of an individual mouse.
B. Expression of transcription factors T-bet, GATA-3, RORγ and Foxp3 were compared between the two mouse strains.

into genes related to the innate and adaptive arm of the immune system. Supportive Figure 1 shows an interactome chart of these genes, where up-regulated genes are indicated in red and down-regulated genes in blue. Innate immune transcripts up-regulated upon VSL#3 administration include *Nos2* and *Nod2*. In BALB/c mice we also observed modulation of adaptive immune cells, as suggested by an increased expression of *Il-12rb1*, *Ccr5* and *Cxcr3,* as well as the chemokine *Cxcl-10*. On the other hand, *Il-13* and *Epx* were down-regulated in the small intestine of these mice. Remarkably, colons of BALB/c mice showed down-regulation of *Il17a* and *Foxp3*.

In C57BL/6 mice, significant immunomodulatory effects were present both in small intestine and colon, but these could not be linked to a defined immunological process.

A)



B)

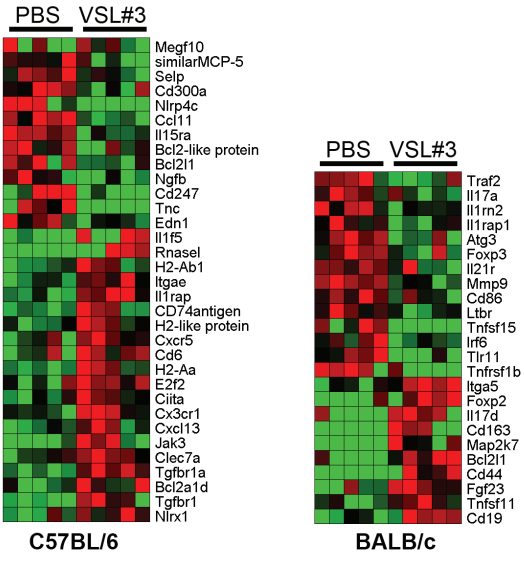


Figure 2. Effect of oral administration of VSL#3 on the expression of genes related to immune function

A heat-map of immune-related genes with significantly different expression in VSL#3-treated mice compared to PBS-treated mice. Relative expression levels for each individual gene are presented as minimum (blue) and maximum (red). Columns represent gene expression profiles of individual mice for the small intestine (A) and colon (B).

Probiotic bacteria modulate local innate and adaptive immune cells in healthy BALB/c mice

As shown above, treatment with VSL#3 influenced the intestinal expression of a variety of genes related to the immune response Moreover, we showed previously that prolonged treatment with VSL#3 rendered BALB/c mice resistant to the induction of colitis (30). Therefore, we evaluated the effect of prolonged treatment on innate and adaptive immune cells in healthy BALB/c mice.

As shown in Figure 3, prolonged treatment with VSL#3 was associated with decreased numbers of $CD4^+$ T cells ($p<0.01$), in conjunction with increased numbers of $CD19^+$ B cells ($p<0.05$), in PP and MLN, but not in spleen. $CD8^+$ T cells were unaffected. Similar results were obtained in C57BL/6 mice ($p<0.01$, data not shown).

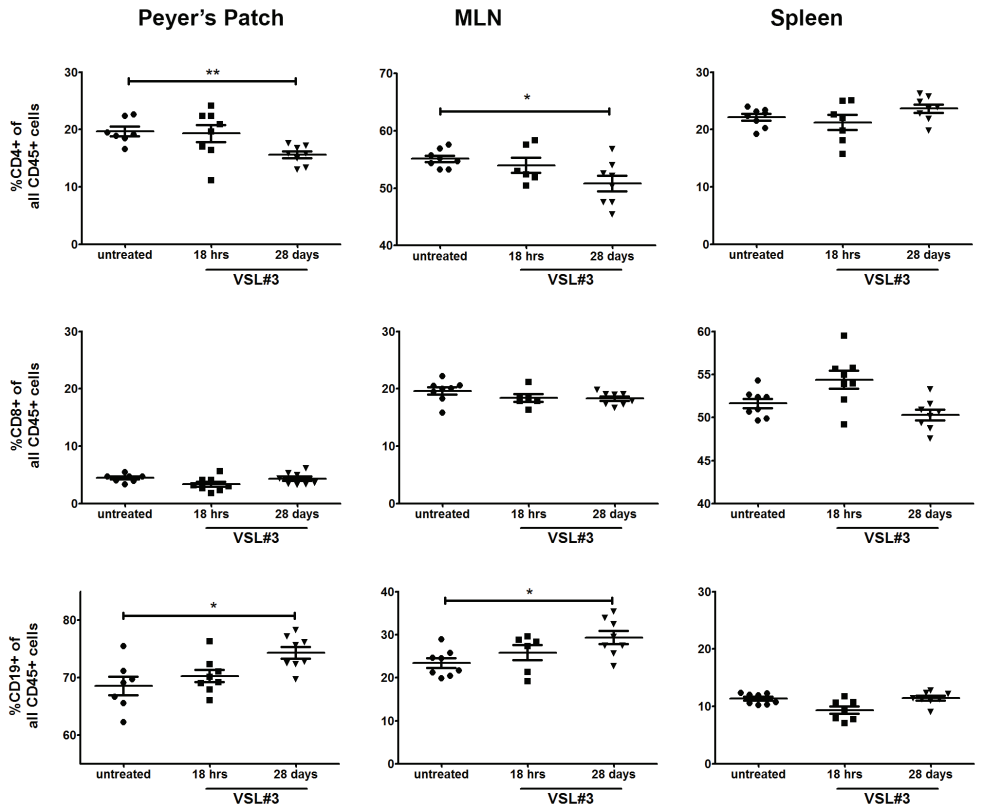


Figure 3. Prolonged administration of VSL#3 alters B and T cell frequencies in PP and MLN
Single cells suspension of Peyer's Patches and mesenteric lymph nodes were prepared from BALB/c mice 18 hours after one single dosage of VSL#3, or after a treatment period of 4 weeks. Cells from untreated mice served as a control.

For BALB/c, we also evaluated effector and regulatory T cells. Figure 4 shows that prolonged VLS#3 treatment was associated with an increase of IL-17$^+$ Th17 cells ($p<0.05$) as well as of Foxp3$^+$ T reg cells ($p<0.01$), whereas numbers of IFN-γ$^+$ Th1 cells remained unchanged. At early time points, i.e. 18 hours after one single dosage of VSL#3, no such effects were observed.

Evaluation of innate cells showed an increase of CD11c$^+$ cells in PP ($p<0.01$) and an increase of F4/80$^+$ macrophages ($p <0.01$) in MLN after prolonged exposure to VSL#3 (Figure 5). Remarkably, a subset of CD11c$^+$ cells expressing CD103 - and reported to induce regulatory T cells (34, 35) was transiently increased both in PP an MLN ($p<0.05$ and $p<0.001$, respectively), 18 hours after the first single dosage of VSL#3.

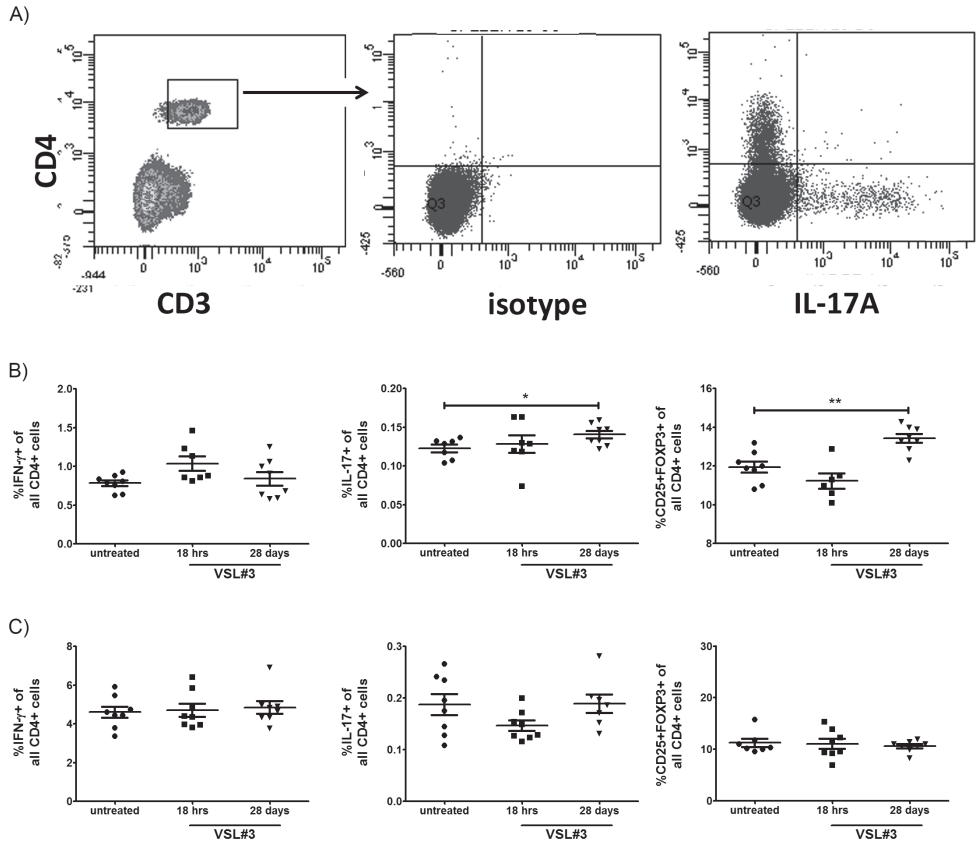


Figure 4. Effect of VSL#3 treatment on the development of effector T cells
MLN cells from BALB/c mice were analyzed after 4 weeks of treatment with VSL#3. Cells were stained for CD3 and CD4, fixated and permeabilized prior to detection of intracellular IL-17 and IFN-γ. A representative FACS plot of cells stained for intracellular cytokines is shown in Figure 4A. IFN-γ$^+$, IL-17$^+$ and Foxp3$^+$ cells are depicted as a percentage of CD4$^+$ cells in MLN and spleen (Figures 4B and 4C, respectively). Results are depicted as mean ± SD for 8 individual mice per group. Statistical significance compared to vehicle treated mice was calculated using the Mann-Whitney U test. *, $p < 0.05$; **, $p < 0.01$.

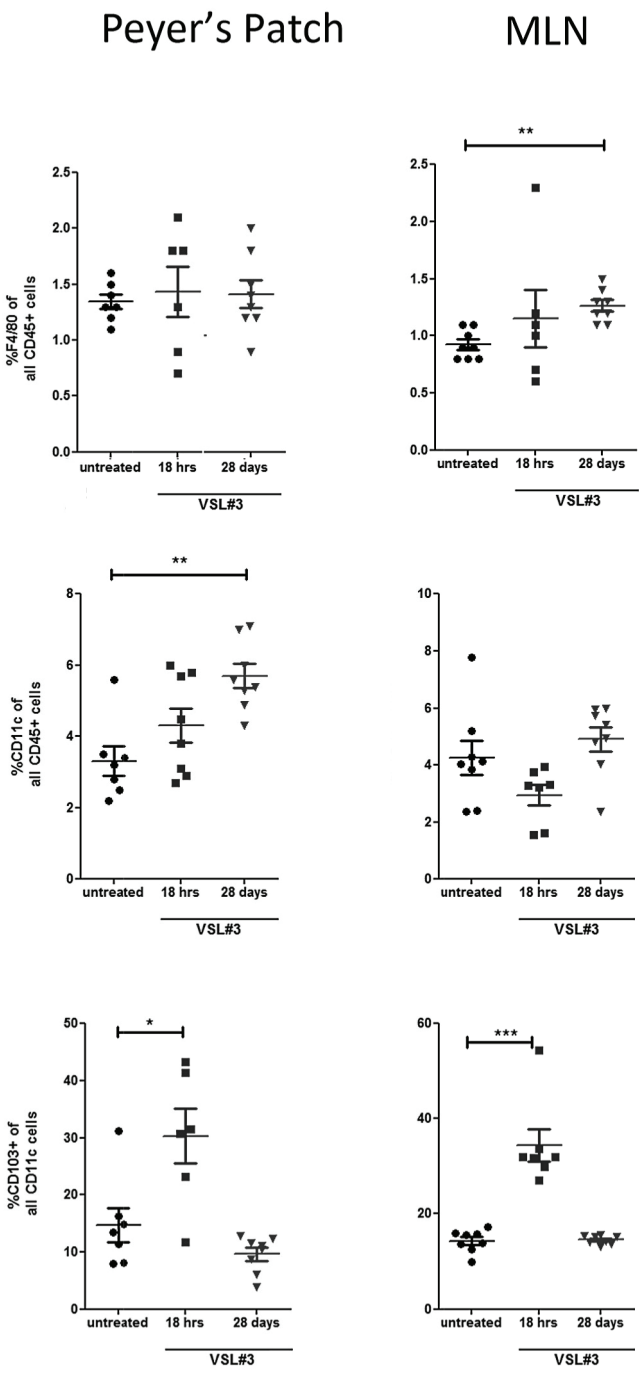


Figure 5. VSL#3 treatment has immediate effect on CD103+ DC

Single cells suspension of Peyer's Patches and mesenteric lymph nodes were prepared from BALB/c mice, 18 hours after one single dosage of VSL#3 or after a treatment period of 4 weeks. Cells from untreated mice served as a control. Cells were analyzed by flowcytometry. F4/80$^+$MΦ and CD11c$^+$ DC are expressed as a percentage of CD45$^+$ mononuclear cells. CD103$^+$ DC are expressed as a percentage of the total CD11c$^+$ population. Results of individual mice are shown. Mann-Whitney U test, * $p < 0.05$, ** $p < 0.01$.

Discussion

In a previous study we showed that prophylactic treatment of mice with the probiotic supplement VSL#3 renders BALB/c mice resistant to the induction of colitis (30). Moreover, VSL#3 and various related *Lactobacillus* and *Bifidobacteria* species have been shown to alter phenotype, cytokine and chemokine release in both human and mouse DC (36, 37). We recently showed that VSL#3 inhibits LPS-induced activation of mouse and human DC especially by dampening the expression and secretion of chemokines (Mariman et al, *submitted for publication*). Modulation of chemokines by probiotics may thus result in a diminished recruitment of immune cells to the intestines under inflammatory conditions. Here, we show that this probiotic mixture also modulates immune cells in conditions of homeostasis.

The intestinal microbiota contributes to the development of the immune system, and conversely, the immune system influences the composition of the microbiota (38, 39). Paneth cell derived defensins, innate antimicrobial peptides that contribute to the mucosal host defense, play a role in this respect by affecting the composition of the intestinal bacterial microbiota (40, 41).

We observed that BALB/c and C57BL/6 mice differed with respect to gene expression in colon and small intestine; differentially expressed genes comprised genes related to immune regulation as well as anti-microbial peptides. These findings may not only be due to the genetic background and polarized immune response of these two mouse strains but also to a different composition of the intestinal microbiota of these mice. The assessment of the exact contribution of each of these phenomena therefore requires further studies in germfree animals.

Regardless of these differences in gene expression between the two mouse strains, we observed significant effects of treatment with VSL#3, with emphasis on immune-related processes. Whereas these effects in C57BL/6 could not be linked to a defined immunological process, treatment of BALB/c mice with VSL#3 showed up-regulation of *Il12rb1, Ccr5, Cxcr3 and Cxcl10* and down-regulation of *Il13 and Epx*. This suggest a shift from a Th2 to a Th1 phenotype in BALB/c mice. These data therefore suggest that probiotic treatment may be favorable to control immune responses under polarized conditions.

To verify the effects of probiotic treatment at the level of innate and adaptive immune cells we evaluated such cells in PP, MLN and spleen. Irrespective of the mouse strain studied, the number of $CD19^+$ B lymphocytes in the PP and MLN were significantly increased after long term administration of VSL#3. Most B cells in the intestine are plasma cells that secrete IgA into the lumen (38, 42). IgA is a predominant immunoglobulin in mucosal secretions which serves as a first line of humoral defense at all mucosal surfaces, where binding of IgA to microorganisms reduces their motility and adhesive properties within the mucosal

lumen and its surface (43). Previous studies linked beneficial effects of specific *Lactobacillus* species to increased $CD19^+$ B lymphocytes in the ileal mucosa (44) and increased levels of IgA-producing cells in the lamina propria (45, 46). Therefore, it is tempting to speculate that increased B cell frequencies observed in our study contribute to the beneficial effects of VSL#3 via IgA-mediated mechanisms.

Interestingly, prolonged treatment of BALB/c mice with VSL#3 also resulted in an increase of $CD4^+CD25^+Foxp3^+$ T reg cells locally in the MLN, but not in the spleen. It has been shown that the induction of $CD4^+CD25^+Foxp3^+$ T reg cells in MLN depends on $CD103^+$ DC in the lamina propria (34, 35). Therefore, our observation that this $CD103^+CD11c^+$ DC subset increased in the PP and MLN of BALB/c mice, 18 hours after the first treatment with VSL#3, may explain the increased numbers of T reg cells we found at a later stage in the MLN.

On the other hand, the increase of T reg cells and Th17 cells in MLN may in part be due to migration from the intestine, in view of the decreased expression of *Foxp3* and *Il17a* in the colon.

Recently, we demonstrated by transcriptome profiling of colons that inflammation in the recurrent TNBS colitis model is mediated by a gradual involvement of mast cells (47) and T cells immune processes that are suppressed by treatment with VSL#3 (30). Collectively, our data suggest that VSL#3 treatment has favorable effects in this model by modulating local inflammation through the induction of T reg cells.

The variation between the two mouse strains under investigation i.e. BALB/c versus C57BL/6, was more profound than the effect of the intervention by VSL#3. This is in line with observations in human subjects, where duodenal biopsies were taken before and after intake of a probiotic supplement (48). In this study, transcriptome profiles clustered per person and not per intervention, showing that person-to-person variation in gene expression was the largest determinant of differences between the transcriptomes. Our present data in BALB/c and C57BL/6 mice substantiate these observations. Therefore, a full appreciation of the health benefits of probiotic bacteria with emphasis on translational value should take both genetic and microbial factors into account.

Acknowledgments

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R.M., F.T., F.K. and L.N. designed the experiments; R.M. and F.T. performed the experiments and analyzed the data; R.M., F.K. and L.N. wrote the paper, and L.N. had primary responsibility for final content. All authors read and approved the final manuscript.

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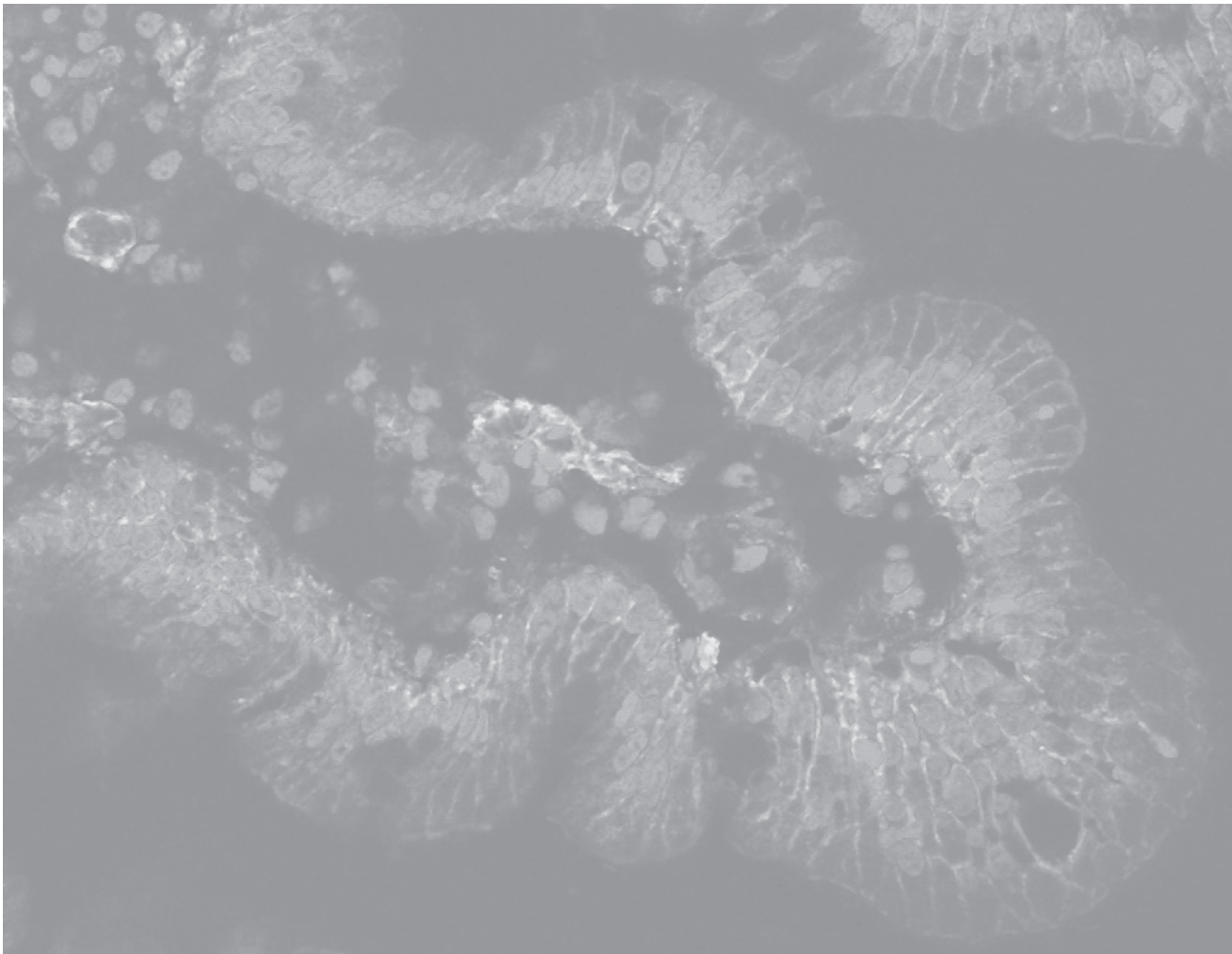
Chapter 8

Summary & General Discussion



The microbiota present in the gastrointestinal (GI)-tract has a fundamental role in the maintenance of host homeostasis [1]. Host-microbiota interactions are of critical importance for tissue formation, metabolism, and the development and function of innate and adaptive immunity [2]. Shifts in the composition of the microbiota induced by improved hygiene, antibiotic treatment, dietary changes or (invasive) pathogens can perturb immune-regulatory networks [3]. Due to these environmental changes many individuals in industrialized countries are no longer exposed to the microbiota of our evolutionary past [4]. The rapid increase in prevalence of immune mediated disorders - autoimmune diseases, inflammatory bowel disease (IBD) [5,6] and allergies [7,8]- in western societies is thought to be mediated in part by disturbances in host-microbiota interactions [9]. Restoring immune homeostasis by modulating host-microbiota interactions with probiotic bacteria has been demonstrated to be effective in chronic intestinal diseases and allergies [10,11]. Therefore, a better understanding of the mode of action of probiotics may help to advance the development of nutritional and pharmaceutical intervention strategies that may help to reverse the current rise of immune-related disorders. This thesis aimed to provide insight into the role of probiotic bacteria in the regulation of mucosal and systemic immune responses and contribution to suppression of inflammatory diseases. This thesis shows that probiotic bacteria have favorable effects in a model of IBD and provides evidence regarding the underlying mechanism.

Immune regulation in Inflammatory Bowel Disease

IBD patients are currently treated with anti-inflammatory and immunosuppressive drugs, antibiotics, surgery and/or biologicals such as anti-TNF[12]. Monoclonal antibodies directed against TNFα have substantially improved therapy, although a considerable proportion of the patients is non-responder, develops neutralizing antibodies after prolonged use, or experiences serious side effects [13-16]. Classical immunosuppressive drugs and antibiotics have rather limited efficacy, and show adverse side effects. So there is a clear medical need to develop novel anti-inflammatory treatments for IBD patients; this may include the use of probiotic bacteria. Probiotics with potential health promoting effects could most probably be validated through the use of proper animal models.

One of the key issues with respect to animal models is their translational value for patients. Chapter 2 describes a model based on the previous published recurrent TNBS-induced colitis model [17,18]. We characterized the processes associated with the early stages of colitis by genome-wide transcriptome analysis of colon tissue. TNBS-induced colitis has originally been positioned as a model for Crohn's disease (CD), based on histopathological features like transmural cellular infiltrations and local production of T_h1/T_h17 cytokines [19,20]. When

compared with recently published gene expression data from inflamed biopsies from ulcerative colitis (UC) [21] and CD [22] patients, the recurrent TNBS colitis model overlapped respectively 18 and 16 percent. This overlap is comparable with other genome-wide transcription studies aimed to identify genes and biological processes affected in experimental colitis, i.e. acute TNBS colitis model in rats [23], murine DSS colitis [24], IL-$10^{-/-}$ colitis [25], and $CD4CD45Rb^{hi}$ T-cell transfer colitis [26].Yet, pathway analysis identified major processes important for IBD i.e. tissue morphogenesis, wound healing, immune/inflammatory response, cell adhesion and angiogenesis. The model therefore represents several aspects of mucosal inflammation in UC and CD patients. Indeed, expression of α-defensins and chemokines as well as the influx of macrophages, T-lymphocytes and mast cells in the intestinal mucosa represents aspects of mucosal infiltration in UC and CD. Moreover, local budesonide treatment, a corticosteroid used to treat IBD patients[27,28],partly reduced colitis features in this model.
A hallmark of chronic inflammatory intestinal disorders is the rapid recruitment of leucocytes, in particular T cells, to the site of inflammation [29]. Mediators that facilitate these processes include adhesion molecules and chemokines [30], and these were induced in the model particularly 2 days after each TNBS challenge. Recent studies indicate that targeting these molecules may be promising for the treatment of IBD [31-33]. Novel compounds evaluated in this respect include Natalizumab(anti-α_4) [31,34,35], Vedolizumab (anti-β_7) [36,37] , ISIS2302 (anti-ICAM1) [38], and CCX282-B (anti-CCR9). However, targeting these leucocyte migration markers, which are not gut specific, may also raise safety issues [39]. Since the microbiota is involved in shaping the intestinal immune system, i.e. by targeting leucocytes and their migration mediators, modulating the microbiota might be a new treatment approach/ strategy in individuals susceptible for intestinal inflammation. The study described in chapter 3 shows that recurrent TNBS colitis model is sensitive to probiotic bacteria. Possible mechanisms underlying these beneficial effects will be discussed in the next paragraph.

Modulating mucosal immune responses by probiotic bacteria

To gain insight into the anti-inflammatory potential of probiotic bacteria the probiotic mixture VSL#3 versus a single strain-*L.plantarum-,* were evaluated by studying their effect on gene expression profiles in the colon. In contrast to studies with these probiotic bacteria in immune compromised mice such as the IL-$10^{-/-}$[25]and T cell transferred $Rag2^{-/-}$ colitis model [40], our results in the recurrent TNBS colitis model reflect immune modulation in mice with a fully functional immune system. Probiotic treatment reduced colitis associated influx of T-lymphocytes, mast cells and macrophages. This was accompanied by suppression of chemokines like MCP-1,eotaxin-1, and eotaxin-2. These chemo-attractants have been implicated in the recruitment of a variety of innate and adaptive immune cells [30,41] and

can be produced by intestinal epithelial cells or innate immune cells in the colonic mucosa. Therefore, it is tempting to speculate that probiotics ameliorate experimental colitis in this model by interfering with these chemokine-secreting cells which consequently results in reduced influx of inflammatory cells.

The induction of regulatory T cells (Treg) represents another potential mechanism by which oral administration of VSL#3 may render mice resistant to TNBS induced colitis. As described in chapter 7, prolonged administration of VSL#3 drives the induction of these FoxP3$^+$Treg locally in the MLN of healthy BALB/c mice. Such cells have the capacity to migrate from a secondary lymphoid organ like the MLN to the inflamed mucosa and suppress T effector cell responses [42].

In IBD, dysregulated T cell responses perpetuate the disease and the vicious cycle of chronic inflammation [43]. It is unknown whether overabundance of T effector cell responses in IBD are the result of reduced numbers of Treg, dysfunction of Treg or resistance of T effector cells for suppression. Apart from a small sub-population of IBD patients with reduced Treg activity caused by a mutation in FoxP3 [44,45], most IBD patients have normal numbers of these cells in the inflamed mucosa [46]. Some reports even show an increase in numbers of Treg cells during inflammation [47-49] possible as a compensation mechanism in an attempt to control inflammation. Since the induction of Treg by probiotic bacteria is considered as a mechanism by which they induce anti-inflammatory effects, prophylactic and therapeutic treatment may have different outcomes. Treg can prevent disease development in spontaneous and chemical induced colitis [50,51]. In immune-compromised mice, adoptive transfer of Treg has also been shown to be efficient in amelioration of an established inflammation [52]. In contrast to chemical-induced colitis models, were Treg lost their potency to suppress colitis when these cells were transferred to mice with established colitis [51]. This might be related to environmental factors, since Treg cells have reduced suppressive capacity if pro-inflammatory cytokines or co-stimulatory signals are present in the inflamed site as shown in various experimental models for autoimmune diseases. [53-55].This implicates that induction of Treg by probiotics could be effective in a prophylactic setting but would fail in IBD patients during periods of active inflammation. Although this is beyond the scope of this thesis, it would be interesting to evaluate the therapeutic potential of probiotics in the recurrent TNBS colitis model.

Modulation of cytokine and chemokines release by probiotics

In chapters 3 and 7it is shown that probiotic bacteria modulate expression patterns of inflammatory mediators like cytokines and chemokines *in vivo* under conditions of inflammation and homeostasis. Probiotic bacteria have been shown to interact with innate

immune cells and intestinal epithelial cells via binding to pattern recognition receptors [56], thereby modulating inflammatory signaling pathways[57]. In chapter 5, *in vitro* studies with bone marrow derived dendritic cells (BM-DC)were employed to evaluate the effects on probiotics on the expression and release of these inflammatory mediators. Studies described in this chapter showed that immune modulating capacities of probiotics on DC depend on host genetics and the presence of other stimulatory signals. DC derived from the bone marrow of two polarized mouse strains i.e. C57BL/6 and BALB/c mice responded differently to ultrapure Toll-like receptor ligands that mimic microbe-associated molecular patterns(MAPS) on bacteria, fungi, and viruses. Host specific responses of BMDC stimulated with these ultrapure ligands that trigger one single receptor, underline the impact of genetic factors on MAMP responses in the gut, but underestimate the complex environment of the gut. DC in the lamina propria(LP) integrate signals simultaneously received from the commensal microbiota and (potential) pathogenic microorganisms. Moreover, during active intestinal inflammation that is often accompanied by impaired barrier function, intestinal DC are exposed to an enormous load of Gram-negative bacteria that reside in the lumen [58]. In chapter 5 it is shown that probiotic bacteria synergistically enhance the production of IL-12p70 and IL-23 in DC from C57BL/6 mice induced by LPS (as a representative component of Gram-negative bacteria). Previous studies have shown that simultaneous activation of TRIF and MyD88 coupled TLR (by bacteria and/or pure ligands) induces a robust induction of IL-12p70 [59]. It is therefore tempting to speculate that Gram-positive probiotic bacteria may induce robust T_h1 and T_h17 responses in individuals with established inflammation. This implies a risk of probiotics use under these conditions, because steering the immune system towards a T_h1/T_h17 inflammatory response could be harmful to patients suffering from T_h1/T_h17-mediated (autoimmune) disease such as CD.

Apart from this pro-inflammatory effect, probiotic bacteria induced anti-inflammatory effects with respect to chemokine expression and release. Irrespective of the mouse strain, VSL#3 and *L .plantarum* suppressed LPS induced up-regulation of CXCL-9 and CXCL-10. These chemokines target the CXCR3 chemokine receptor that is expressed on T_h1 cells, CD8+ T cells and DC [60]. Both chemokines have been shown to play a role in a variety of autoimmune diseases and infectious diseases by recruitment of inflammatory cells to the site of inflammation [61-65]. Moreover, therapy with anti-CXCL-10 antibody reduced experimental colitis in IL-$10^{-/-}$ mice by interfering with recruitment of T effector cells [66,67]. Chapter 6 describes comparable *in vitro* studies with human monocyte derived DC. With the use of dedicated PCR arrays for cytokines and chemokines, the gene expression signature of human DC in response of LPS and VSL#3 was identified. This unbiased approach, not focusing on one single inflammatory mediator, identified a cluster of LPS-induced genes that was suppressed by VSL#3. Chemokines present in this cluster did not target a single receptor, but a diversity of chemokine receptors including CCR8, CCR1, CCR3, CCR7 and CXCR3.

Consequently, this reduced chemokine expression may potentially dampen attraction of a broad panel of immune cells including T cells, NK-cells, DC, B cells, eosinophils, basophils and mast cells [68]. *In silico* analysis of transcription factors appeared a powerful approach to identify a common regulator of LPS-induced genes that were blocked by probiotics. Transcription factor analysis could be used because a large panel of chemokines and cytokines was under investigation in this study. The majority of these inflammatory mediators was not affected by probiotic treatment and could therefore be used as a reference set. STAT-1 was predicted as a dominant driver of LPS-induced genes that were suppressed by VSL#3, which was confirmed by demonstrating that phosphorylation of this transcription factor was inhibited by probiotic bacteria. This suggests that VSL#3 can reduce production of specific chemo-attractants *in vitro* by selective inhibiting of STAT-1 induced transcription regulation. STAT-1 is relevant in intestinal disease, since STAT-1 deficiency reduced the intensity of spontaneous and chemical induced colitis [69,70]. Furthermore, a recent report showed that selective regulation of STAT-1 signaling in T-cells by fusaruside resulted in reduced production of pro-inflammatory cytokines and improved TNBS induced colitis [71]. Understanding how MAMPs present on probiotic bacteria modulate intracellular signaling induced by pathogenic bacteria could be helpful to identify mechanisms by which probiotics promote health *in vivo*. Accordingly, a better knowledge of the molecular mechanisms of induced signaling pathways could allow selection of probiotic strains with distinct properties, leading to more specific and efficacious therapeutic strategies in the prevention or treatment of allergic and autoimmune diseases.

Probiotics modulate immune responses outside the gut

Commensal bacteria influence local immune responses in the gastrointestinal mucosa under inflammatory and homeostatic conditions[10]. However, immune modulation by the GI-microbiota is not restricted to the intestine and may influence immune responses in peripheral tissues as well [8]. Epidemiological studies found strong correlations between altered fecal microbiota and atopic diseases. Atopic diseases, such as asthma and AD are the result of an inflammatory reaction triggered by a T_h2 cell mediated immune response.
In chapter 4,it is shown that there is an association between gut inflammation and atopic dermatitis (AD) in human ApoC1+/+ transgenic mice. Interestingly, transcriptome profiling of the colon further indicated that these mice have reduced intestinal chemokines and cytokines that are associated with T_h1 immune responses along with increased allergic markers like IgE^+ and $Fc\varepsilon RI^+$ cells, suggesting a T_h2 driven inflammation in the intestine.
Recent evidence shows that the commensal microbiota programs many aspects of the adaptive immune system, including T cell responses [75]. Environmental changes such as

antibiotics, diet and infant feeding regimes have been shown to influence the composition of the microbiota [76-79]. It is hypothesized that these changes in the gastrointestinal microbiome composition contribute to the increase in prevalence of immune mediated disorders. Studies in experimental models for rheumatic arthritis [80], experimental autoimmune encephalomyelitis [81]and various animal colitis models demonstrate that the commensal microbiota contributes to autoimmune and allergic disorders at sites distal to the intestinal mucosa[82]. Exacerbation of arthritis and EAE is likely the consequence of pathogenic T_h17 cells that traffic out the LP to their target tissue [80,81,83]. Other studies also indicate that T-cell activation in the intestinal mucosa by microorganisms influences systemic immunity[75]. For example *B. fragilis* PSA affects the nature of systemic T cell responses evident from increased circulating T_h1 cells in mice colonized with this bacterial strain[84].Likewise, it has been shown that *Clostridium IV* and *XIV* in the GI-tract not only induce expansion of Treg in the LP, but also in the periphery[85,86].

Interestingly, oral administration of *L. plantarum* result in amelioration of skin pathology in the human $ApoC1^{+/+}$ transgenic mice model for atopic dermatitis (AD) as shown in chapter 4. This might be due to the induction of local inflammatory responses by this specific *L. plantarum* strain as observed in an *ex vivo* study employing human colon explants [87]. Moreover, oral administration of this lactobacillus strain to healthy mice increased the frequency of IFN-γ positive T cells locally in the mesenteric lymph nodes, as well as in peripheral immune tissue like the spleen [88]. The beneficial effect of probiotics intervention in this mouse model was not limited to the skin. *L. plantarum* also reduced the number of mast cells in the intestinal mucosa. Therefore, it is tempting to speculate that *L. plantarum* exerts the beneficial effect in this mouse model for AD by balancing $T_h1/_{Th2}$ responses. Failure of the probiotic mixture VSL#3 in preventing skin pathology suggests that different strains may act differently in diverse pathological conditions. This implicates a careful selection of probiotic strains for therapeutic use is required, based on the type of inflammation.

Concluding remarks

A better understanding of host-microbiota interactions in the pathogenesis of (auto)-immune and infectious diseases may contribute to new treatments of inflammatory diseases. Manipulating the enteric microbiota with fecal microbiota transplantation and probiotics might have great potential for treating IBD[89-91]. However, to fully benefit from the potential of such strategies deeper understanding of the immune-modulating mechanisms of these interventions is required.

We and others have shown that oral administration of specific probiotic strains reduce the severity of intestinal inflammation and atopic dermatitis in experimental animal models.

However, to improve the application of probiotics in healthy and diseased individuals it is important to define the proper probiotic bacterial strains for a specific therapeutic application. In addition, we showed in chapter 4 and 7 that effects of probiotics on immune related gene expression in the intestine largely depend on host genetics. This is in line with clinical studies, where human biopsies from healthy volunteers taken before and after probiotic intervention also showed large intra-individual differences in their response to probiotics. Therefore, it seems doubtful that all individuals experience similar physiological benefits of a certain probiotic strain. Current probiotics have not been selected for specific treatment purposes but they rather are “all purpose” probiotics. The development of a new generation of probiotic strains for disease specific application therefore may benefit from detailed knowledge of their immune modulating properties. Design of probiotic therapies should be based on the abnormalities in the microbial composition, the underlying genetic defect and desired biological response.
Due to their long history of use in food formation, the FDA has designated most probiotics to be generally recognized as safe (GRAS-status). However, it should be noted that a concern regarding the safety of using probiotics has recently been raised due to increased risk of mortality in patients with severe acute pancreatitis [92]. We showed that the involvement of other immune activating signals could also affect the innate immune responses towards probiotics. This implicates that patients with an established intestinal inflammation may respond differentially to probiotic intervention than healthy individuals. Consequently, it will be necessary to monitor safety issues when probiotics are used to treat IBD patients.

In conclusion, it was shown that immune-modulating properties of probiotic bacteria are strain specific and depend on host genetics and environmental factors. This illustrates the likelihood that personalized probiotic therapies for individuals should be designed, that are based on host genetic polymorphism and the desired biological response rather than non-discriminating heterogeneous patients with a single approach. Improved knowledge about the working mechanisms of probiotics will contribute to proper strain selection for a specific prophylactic or therapeutic application, ultimately leading to more personalized medicine.

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Chapter 9

Nederlandse Samenvatting

Alle verschillende micro-organismen in onze darm, worden samen het “darm-microbioom” genoemd. Het aantal bacteriën in onze darmen is meer dan 10 keer zo groot dan het totale aantal cellen in het menselijk lichaam. Het darm-microbioom beïnvloed diverse processen in de gastheer waaronder fysiologische en immunologische processen.
Veranderingen in de samenstelling van het darm-microbioom in onze huidige samenleving kunnen worden veroorzaakt door een verbeterende hygiëne, het gebruikt van antibiotica en een veranderd dieet. Door deze veranderingen in onze leefomgeving worden we niet langer blootgesteld aan hetzelfde microbioom als onze voorouders. Er wordt gesteld dat de toename van diverse immuun gerelateerd ziekten, waaronder auto-immuun ziekten, chronische darmontsteking (IBD) en allergieën deels wordt veroorzaak door de verstoring van de interacties tussen de gastheer en het microbioom. Het herstellen van de balans in het immuunsysteem door het moduleren van deze interactie tussengastheer en microbioom door middel van probiotische bacteriën is effectief gebleken in de behandeling van Colitis ulcerosaen allergieën. Probiotica zijn door de Wereldgezondheidsorganisatie gedefinieerd als “*Levende micro-organismen, die wanneer in voldoende hoeveelheden toegediend, een gunstig effect hebben op de gastheer”.*
In dit proefschrift is gepoogd een beter inzicht te krijgen in de rol van microbioom-gastheer interacties in de regulering van het immuun systeem.

In hoofdstuk 2 wordt een muismodel voor IBD geïntroduceerd, het zogenaamde TNBS-colitis model. Door in de tijd te kijken naar het expressieprofiel van meer dan 27.000 genen zijn onderliggende pathologische processen in dit colitis model gekarakteriseerd. Hieruit bleek dat dit model wordt gekenmerkt door zowel acute ontsteking als chronisch processen van celinfiltratie, angiogenese en fibrose, welke allen relevant zijn voor humane pathologie. Daarnaast is ook aangetoond dat immuun-suppressieve medicijnen de ernst van dedarmontsteking verminderen, wat het model geschikt maakt voor het testen en ontwikkelen van nieuwe medicijnen.
In hoofdstuk 3 is dit *in vivo*model gebruikt om te evalueren of probiotische bacteriën een gunstig effect hebben op ontsteking in de darm. Muizen behandeld met de probiotsche stam *Lactobacillusplantarum*of de commercieel beschikbare mix van probiotische bacteriën VSL#3 waren deels beschermd tegen de inductie van ziekte. Een mogelijk verklaring voor dit gunstige effect is gelegen in het feit dat VSL#3 de gen-expressie van chemokines-signaalstoffen verantwoordelijk voor de rekrutering van immuuncellen - onderdrukt.

Diverse studies met proefdieren tonen aan dat voeding met probiotische bacteriën een allergische reactie in de huid kan verminderen. Daarom is in hoofdstuk 4 gekeken naar de relatie tussen immunologische processen in de darm en de huid. Hiervoor zijn transgene ApoC1 muizen gekozen, die in de huid spontaan atopische dermatitis (atopisch eczeem)

ontwikkelen. In vergelijking met normale muizen hebben deze transgene muizen in de dikke darm meer Mest-cellen en een verhoogd aantal cellen die de allergische markers IgE en FcεRI^{+} tot expressie brengen. Toediening van de probiotische stam *Lactobacillus plantarum* verlaagde het aantal Mest-cellen in de dikke darm en verminderde de symptomen van atopische dermatitis. Dit suggereert dat beïnvloeding van het immuun systeem in de darm kan bijdragen aan het onderdrukken van atopische dermatitis in de huid.

Om meer inzicht te krijgen in de manier waarop probiotische bacteriën het immuun-systeem beïnvloeden is gebruikt gemaakt van celkweek systemen met dendritische cellen. Deze dendritische cellen zijn onderdeel van het aangeboren immuunsysteem en herkennen bacteriën, virussen en andere micro-organismen via speciale receptoren op het celoppervlak. Door het opnemen en presenteren van antigenen dirigeren ze het adaptieve immuunsysteem waaronder T-cellen. Hoofdstuk 5 worden studies met dendritische cellen uit muizen beschreven. Deze laten zien dat de probiotische mix VSL#3 zowel pro- and anti-inflammatoire eigenschappen kan hebben op dendritische cellen, en dat de genetische achtergrond van de gastheer hierin een belangrijke rol speelt. Vergelijkbare studies met dendrische cellen van humane origine, gepresenteerd in hoofdstuk 6, tonen dat deze effecten gevonden in de muis vertaalbaar zijn naar zijn de mens. In deze studie werd verder aangetoond dat de probiotische mix VSL#3 de fosforyleringvan STAT-1 blokkeert, waardoor de productie van specifieke groep chemokines wordt geremd.
Concluderend, in dit proefschrift werd aangetoond dat het effect van probiotica op het immuunsysteem afhankelijk is van zowel de probiotische stam, het genotype van de gastheer alsmedeomgevingsfactoren. Dit impliceert dat voor een succesvolle toepassing van probiotica in de kliniek rekening dient te worden gehouden met de genetische achtergrond van de gastheer en de gewenste immunologische verandering. Uiteindelijk zal een verbeterd inzicht in de interactie tussen probiotische bacteriën en gastheer kunnen leiden tot de selectie van de juiste probiotische stam(men)voor specifieke profylactischeentherapeutische toepassingen.

Curriculum Vitae

Rob Mariman werd geboren op 22 december 1983 in Tilburg. In 2002 behaalde hij zijn VWO diploma (profiel Natuur & Techniek en Natuur & Gezondheid) aan het Theresia Lyceum in Tilburg. Vervolgens startte hij in september 2002 met de studie Life Science and Technology aan de Technische Universiteit Delft en de Universiteit van Leiden.

In het kader van zijn Masteronderzoek voerde hij zijn eerste onderzoeksstage uit in het laboratorium van dr. E. Vreugdenhil aan het Leiden Amsterdam Centre for drug Research. Hier deed hij onderzoek naar de rol van microRNAs op de expressie van de glucocorticoïde receptor in de hersenen. Ter afsluiting van zijn Master deed hij een bedrijfsstage bij Chiral Vision BV, gericht op de toepassing van enzymen in de organische synthese. Na het voltooien van deze stage behaalde hij zijn Master diploma cum laude.

Na zijn afstuderen werd Rob in september 2008 aangesteld als promovendus bij de afdeling Immunohematologie en Bloedtransfusie van het LUMC. Voor zijn promotie onderzoek werd hij gedetacheerd op de afdeling Metabolic Health Research van TNO in Leiden onder supervisie van Dr. L. Nagelkerken. Het promotie onderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, heeft hij afgerond in 2013.

Momenteel is Rob aangesteld als post-doctoraal onderzoeker aan de vakgroep Animal Genomics aan de Universiteit van Luik, waar hij onderzoek doet naar de rol van microbiota bij het ontstaan van de ziekte van Crohn.

Publications

- Mariman R, Kremer B, Koning F, Nagelkerken L. **Dualistic nature of probiotic bacteria: identification of concomitant pro- and anti-inflammatory effects on mouse dendritic cells.** (submitted)
- Mariman R, Reefman E, Persoon-Deen C, Koning F, Nagelkerken L. **Probiotics modulate intestinal mast cells and atopic dermatitis in human ApoC1+/+ transgenic mice.** (submitted)
- Mariman R, Kremer B, Koning F, Nagelkerken L. **Probiotics dampen TLR-4 mediated activation of human dendritic cells by inhibition of STAT-1 phosphorylation.** (submitted)

– Kremer B, Mariman R, Koning F, Nagelkerken L. **Temporal colonic gene expression profiling in the recurrent colitis model identifies early and chronic inflammatory processes.** PLoS One 2012, 7(11): e50388.
– Liang W, Tonini G, Mulder P, Kelder T, van Erk M, van den Hoek AM, Mariman R, Wielinga PY, Baccini M, Kooistra T, Biggeri A, Kleemann R. **Coordinated and interactive expression of genes of lipid metabolism and inflammation in adipose tissue and liver during metabolic overload.** PLoS One 2013, 25;8(9):e75290.
– Mariman R, Kremer B, van Erk M, Lagerweij T, Koning F, Nagelkerken L. **Gene expression profiling identifies mechanisms of protection to recurrent trinitrobenzene sulfonic acid colitis mediated by probiotics.** Inflamm Bowel Dis. 2012, 18(8):1424-33
– Vreugdenhil E, Verissimo C, Mariman R, Kamphuis J, Barbosa J, Champagne D, Meijer O, de Kloet ER, Fitzsimons C. **MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: implications for glucocorticoid responsiveness in the brain.** Endocrinology 2009, (5):2220-8.

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Stellingen behorend bij het proefschrift

Probiotic bacteria and the immune system: mechanistic insights and therapeutic implications

1. Pathological and immunological characteristics of the TNBS colitis model are not restricted to Crohn's disease, but should be considered as a general model for intestinal inflammation. (this thesis)

2. Probiotics selectively interfere with the production of pro-inflammatory cytokines, which consequently results in reduced influx of inflammatory cells (this thesis)

3. Modulation of intestinal immune homeostasis contributes to the suppression of atopic dermatitis. (this thesis)

4. Probiotic bacteria can exert both pro- and anti-inflammatory effects on DC, and their immune-modulating properties may be influenced by host-genetics and environmental factors. (this thesis)

5. A better understanding of host-microbiota interactions in the pathogenesis of (auto)-immune and infectious diseases may contribute to new treatments of inflammatory diseases. (Elson, et al. Gut Microbes. 2012 (4):332-44)

6. The role of intestinal DC in driving intestinal immune tolerance and capacity of local factors to determine their function make them attractive targets for manipulating immune responses. (Mann, et al. Immunol Lett. 2013 (1-2):30-40)

7. To fully benefit from the potential of probiotic bacteria a deeper understanding of the immune-modulating mechanisms of these bacteria is required.

8. Understanding the molecular mechanisms of action of probiotic bacteria will contribute to better designed probiotic products, designed for a clearly characterized target populations. (Gerritsen, et al. Genes Nutr. 2011 (3):209-40)

9. Not everything that can be counted counts, and not everything that counts can be counted. (Einstein)

10. Nothing is more fairly distributed than common sense: no one thinks he needs more of it than he already has. (Descartes)