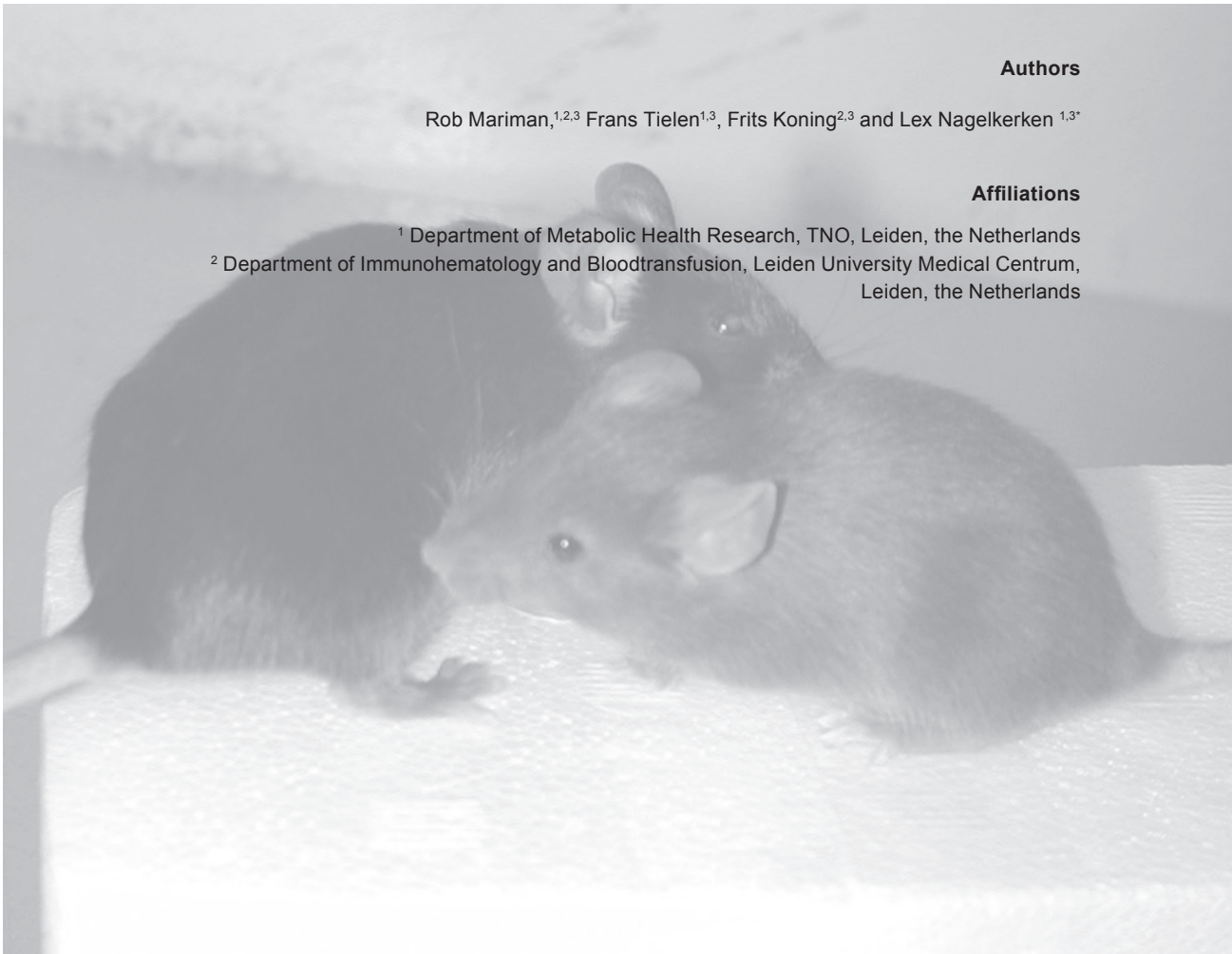
Chapter 7

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

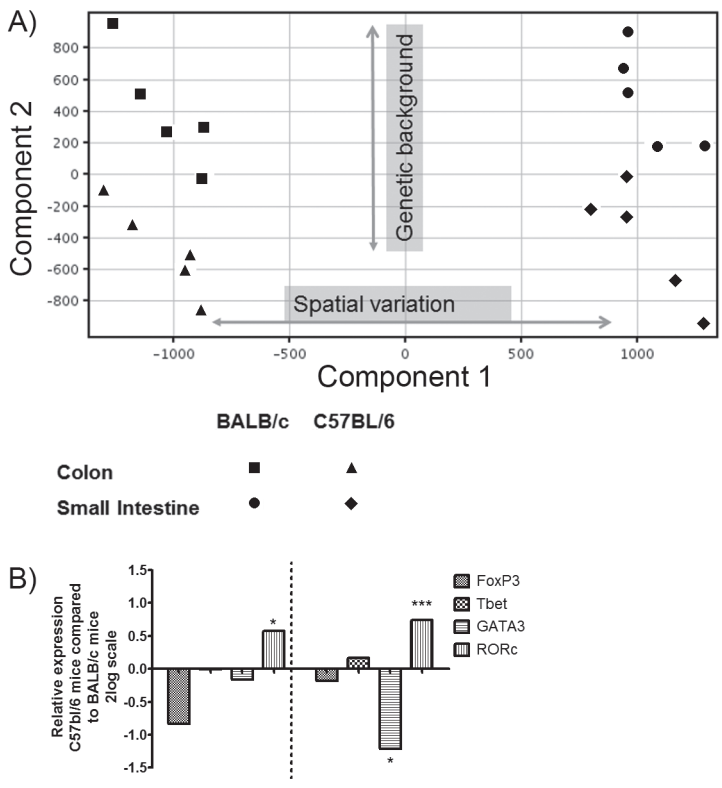
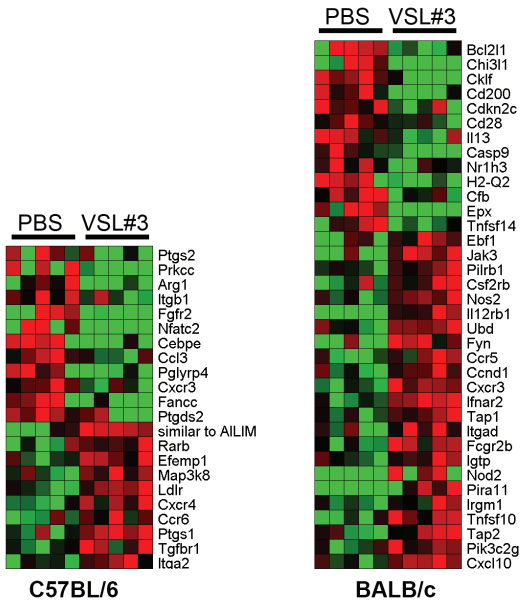


Figure 1. Gene expression profiles in the intestine of BALB/c and C57BL/6 mice
A. Two-dimensional visualization of principal component analysis (PCA) constructed with all 27,000 genes on the array. Each dot represents the expression profile of an individual mouse.
B. Expression of transcription factors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



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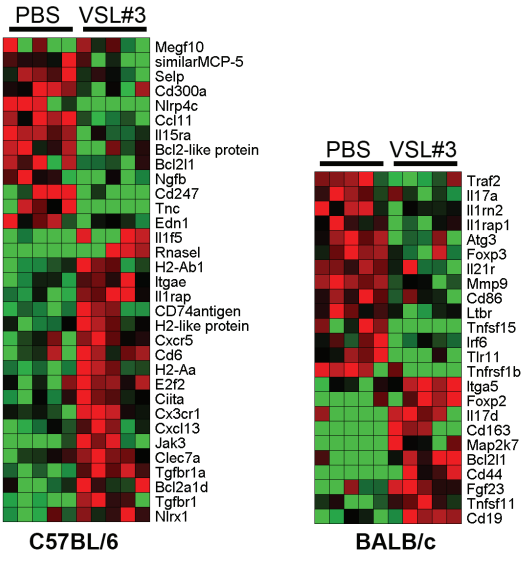

Figure 2. Effect of oral administration of VSL#3 on the expression of genes related to immune function

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).

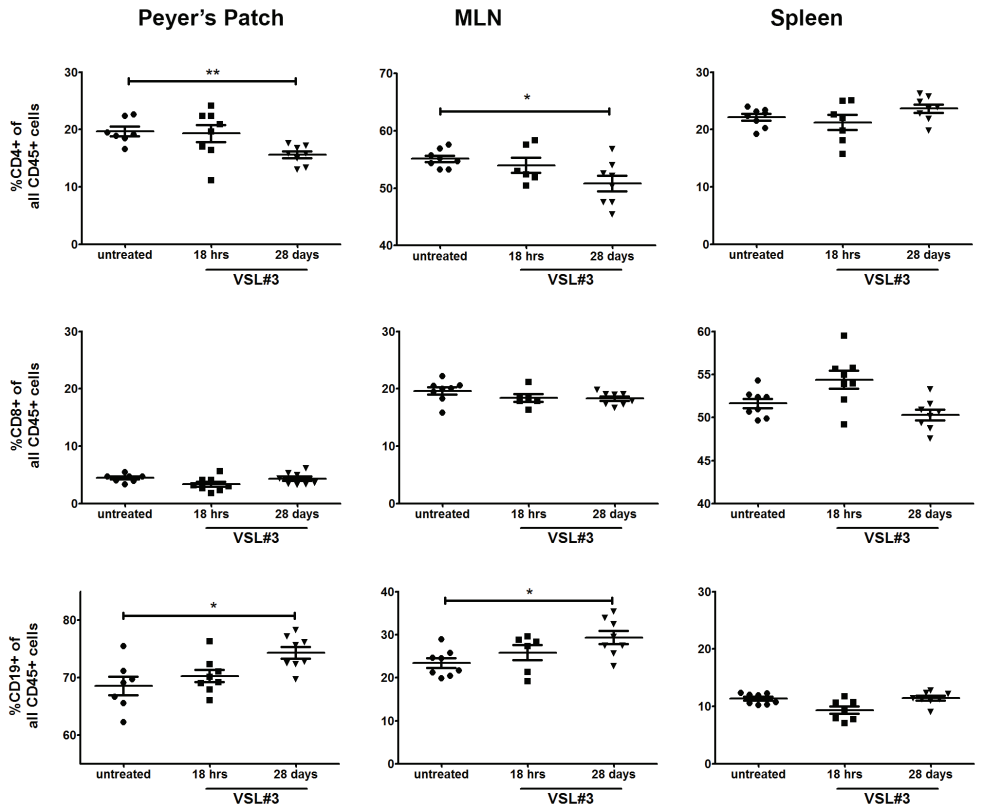

Figure 3. Prolonged administration of VSL#3 alters B and T cell frequencies in PP and MLN
Single cells suspension of Peyer's Patches and mesenteric lymph nodes were prepared from BALB/c mice 18 hours after one single dosage of VSL#3, or after a treatment period of 4 weeks. Cells from untreated mice served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

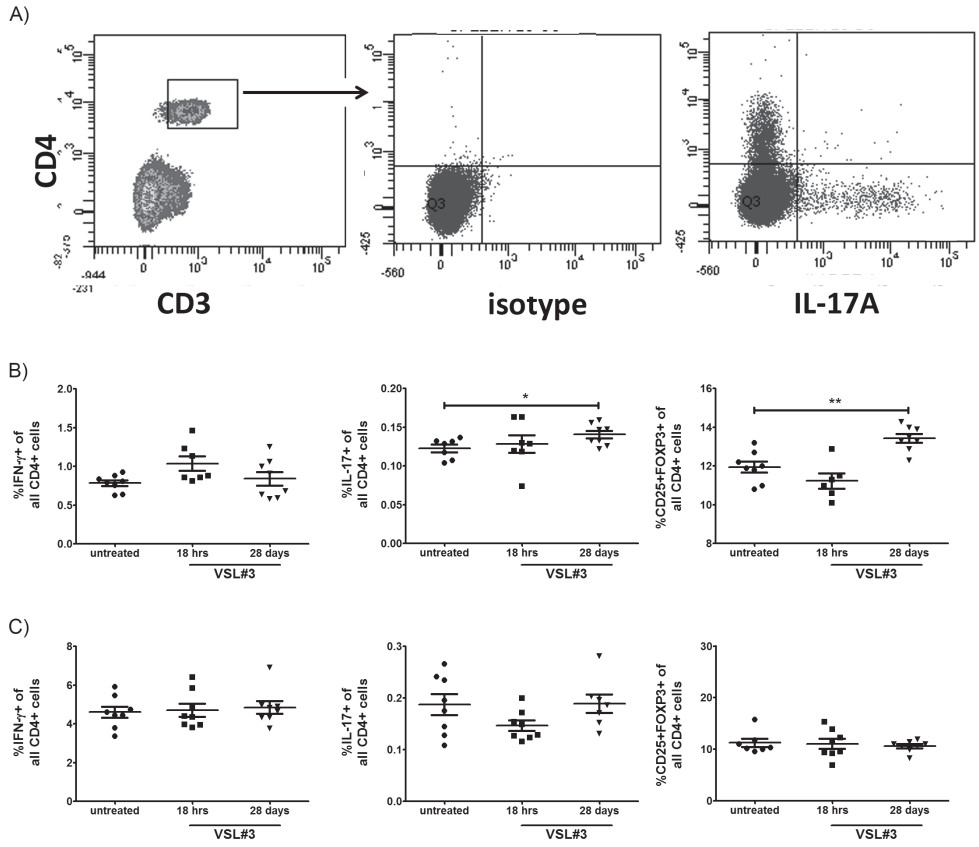

Figure 4. Effect of VSL#3 treatment on the development of effector T cells
MLN cells from BALB/c mice were analyzed after 4 weeks of treatment with VSL#3. Cells were stained for CD3 and CD4, fixated and permeabilized prior to detection of intracellular IL-17 and IFN-γ. A representative FACS plot of cells 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. *, $p < 0.05$; **, $p < 0.01$.

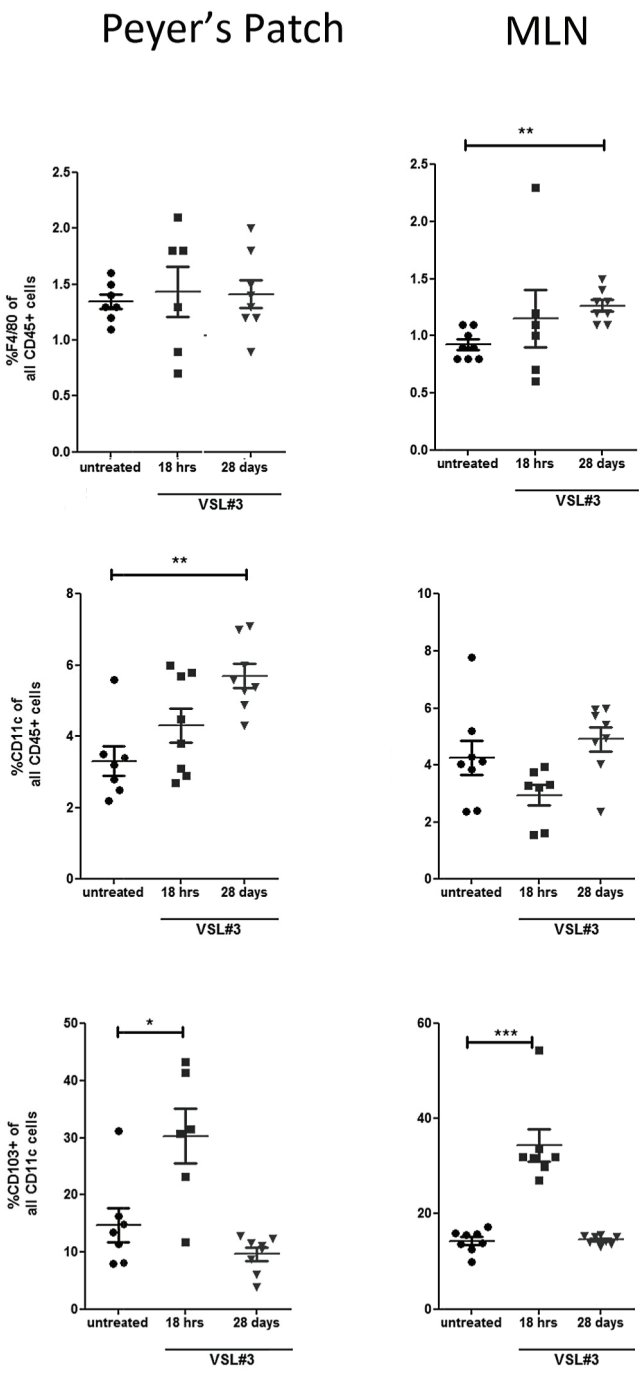

Figure 5. VSL#3 treatment has immediate effect on CD103+ DC

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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