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RESEARCH

Intravenous Administration of a Single-Dose Free-Circulating DNA of Colitic Origin Improves Severe Murine DSS-Colitis

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Abstract In inflammatory bowel diseases the presence of free-circulating DNA (fcDNA) sequences in the sera is an established phenomenon, albeit its real biological function still remains unclear. In our study the immunobiologic effects of a single-dose, intravenously administered fcDNA of normal and colitic origin were assayed in DSS-colitic and control mice. In parallel with disease and histological activity evaluations changes of the TLR9 and inflammatory cytokine signaling gene expression profiles were assayed in isolated cells of the lamina propria. Intravenously administered colitis-derived fcDNA displayed a more prominent beneficial action regarding the clinical and histological severity of DSS-colitis than that of fcDNA of normal origin. Systemic administration of colitis-derived fcDNA significantly altered the expression of certain TLR9-related and proinflammatory cytokine genes in a clinically favorable manner. Presumably due to induction of severe colitis, the subsequent marked inflammatory environment may result changes in fcDNA with a potential to promote the downregulation of inflammation and improvement of tissue regeneration. Elucidating mechanisms of innate immune alterations by nucleic acids may provide further insight into the etiology of inflammatory bowel diseases, and develop the basis of novel nucleic acid-based immunotherapies.

Keywords Free-circulating DNA · DSS-colitis · Toll-like receptor 9 · Proinflammatory cytokines · Tissue regeneration

Introduction

Inflammatory bowel diseases (IBD) result from an inappropriate response of the defective mucosal immune system to intestinal microbiota and other luminal antigens. The traditional treatment modalities for IBD mainly target aberrant immune responses and inflammatory cascades, however, some of them have limited beneficial effect, and may provoke severe side effects [1].

The presence of free-circulating DNA (fcDNA) sequences in the serum is a well-known phenomenon [2–4]. The amount of these sequences may be increased and the quality may be altered within chronic inflammatory disorders such as IBD [5, 6]. Furthermore, a close correlation between the quantity of fcDNA and the course or prognosis of several physiologic (e.g. pregnancy) and pathologic conditions (e.g. sepsis, tumorous- and autoimmune disorders, myocardial infarction) has been described [7]. However, the biological importance and role of fcDNA are still questionable and need to be clarified.

Toll-like receptors (TLRs) are innate immune receptors that detect and clear invading microbial pathogens. Intracellular TLRs (TLR3, TLR7, TLR8, TLR9) specifically recognize nucleic acids such as double-stranded RNA, single-stranded RNA, and CpG DNA derived from microbial components [8]. TLR9 expressing cells in the colonic lamina propria [9, 10]

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usually accumulate within the area of isolated lymphoid follicles (ILFs) and lymphoid aggregates (LAs) [11]. The methylation status of fcDNA may reflect its organ origin [3].

We have chosen dextran sulfate sodium (DSS)-colitis as an experimental mouse model because this model is particularly useful for investigating innate immune contributions to colitis [12]. Moreover, mice with acute DSS-induced colitis exhibit similar cytokine profile expression and histologic changes to human IBD [13]. The useful therapeutic effects of orally administered immunostimulatory DNA sequences and their synthetic oligonucleotide analogs have already been demonstrated in experimental murine colitis [14]. Furthermore, intraperitoneal (ip.) administration of immunostimulatory TLR9-agonist DNA sequences has been found to protect mice from DSS-induced colitis via the induction of indoleamine 2,3 dioxygenase-1 (IDO-1) [15]. Ip. injection is frequently reported to be as effective as intravenous (iv.) injection, but it has been recently reported that ip. DNA analogue administration displays altered pharmakokinetics as compared it to the iv. route [16]. Local administration of a TLR-agonist synthetic oligonucleotide sequence (DIMS0150) in humans has recently demonstrated clinical efficacy by restoring glucocortocoid sensitivity [17], but the repetitive colonoscopy-based therapy administration is difficult. Therefore, in the future, more easier and convenient route of drug administration (orally or parenterally) is required. To the best of our knowledge, the effect of iv. administered self-fcDNA has yet to be examined.

In our present experiment, the biologic effects of iv. administered fcDNA were studied in a DSS-induced murine colitis model. Systemic administration of colitis-derived fcDNA reduced the clinical and histopathologic severity of DSS-induced colitis in mice, as well as altered TLR9-related signaling and the proinflammatory cytokine profile in a favorable manner.

Materials and Methods

Study Design: Induction of Colitis, Isolation and Administration of Free-Circulating DNA

Six-week-old, male C57bl/6 mice were purchased from the 1st Department of Pathology and Experimental Oncology, Semmelweis University, Budapest, Hungary. All mice were housed four per cage and fed standard laboratory chow in the animal room with 12-h dark/light cycles and a constant temperature of 22 °C. Six experimental groups (Group 1–6; G1–6) of animals were established (Table 1).

Experimental colitis was induced by giving mice (Group 2, 4 and 6) drinking water ad libitum containing 5 % (w/v) DSS (mol. wt. approx. 40.000; Sigma-Aldrich) for 5 days.

For the source of fcDNA treatment (i.e. N-fcDNA and C-fcDNA), two separated groups of 6-week-old male mice (No.=50, in each) were used. Group I included normal, non-fcDNA-treated, control animals, while in Group II DSS-colitis was induced as described earlier. Blood samples from Group I and II were collected and pooled separately.

FcDNA was isolated from the plasma of animals by using Qiagen Circulating Nucleic Acid kit (Qiagen, Hilden, Germany). Size range of fcDNA was between 200 bp and 10 kbp according to gel electrophoresis. The average amount of fcDNA in 1 μl of plasma was 28 ng (26–32 ng) in normal (Group I), and 51 ng (42–56 ng; determined by PicoGreen Assay; Invitrogen, Palo Alto, California, USA) in colitic mice (Group II). After isolation and purification of fcDNA from the pooled samples, 200 ng of fcDNA was resolved in 200 μl of sterile saline solution (0.9 % of NaCl). No protein, RNA or lipopolysaccharide contamination was detected in the purified fcDNA. In Groups 3, 4, 5 and 6 mice were injected intravenously via the tail vein with a single-dose of fcDNA on day 5 of DSS treatment. Fourteen days were given for regeneration, then mice were sacrified on day 19. The schematic view of our study can be seen on Fig. 1.

Macroscopic Analysis: Assessment of Inflammation

The body weight of the animals was recorded at day 0, day 5, and day 19. During the experiment, none of the mice died nor lost more than 25 % of body weight. At the time of sacrifice (day 19), a careful colonic preparation between the ileocecal junction and the proximal rectum was performed and presence of loose stool and visible fecal blood was assessed. At the same time, disease activity index (DAI) was established in each of the cases [18]. Briefly, body weight loss was scored on a scale of 0–4 (0: <1 %; 1: 1–5 %; 2: 5–10 %; 3: 10–15 %; 4: >15 %). Loose stool was scored on a scale of 0–4 (0: normal; 1: loose droppings; 2: loose stools, colon filled with feces; 3: loose stools, feces only near cecum; 4: empty bowel). Visible fecal blood was scored on a scale of 0–4 (0: negative; 2: positive; 4: gross bleeding). DAI was determined by the combination of the above three scores, as described earlier [18, 19]. Colon length was also recorded and used as an indicator of disease-related intestinal wall thickening. For macroscopic and microscopic evaluation, other parenchymal organs (such as the stomach, small bowel, kidneys, spleen, lungs, liver and pancreas) were also removed and analysed. Macroscopic evaluation involved the registration of the weight/length/diameter of the organs.

Histological Analysis

Colons were cut longitudinally into two parts. From the terminal, most inflamed part of the colon, a piece of 20 mm was placed into RPMI-1640 medium (R1145, Sigma-Aldrich) for isolation of lamina propria (LP) lymphocytes. Other colonic segments and other parenchymal organs were fixed in 10 % of

Table 1 Characteristics of the animal groups in the experiment

	5 % of DSS administration	Treatment with fcDNA from *normal* mice (*N*-fcDNA)	Treatment with fcDNA from DSS-*colitis* mice (*C*-fcDNA)
G1 mice: absolute control (no-DSS; no-fcDNA treatment; no.=6)	−	−	−
G2 mice: DSS-colitic; no-fcDNA treatment (no.=6)	+	−	−
G3 mice: non-colitic; normal fcDNA treatment (no.=12)	−	+	−
G4 mice: DSS-colitic; normal fcDNA treatment (no.=12)	+	+	−
G5 mice: non-colitic; colitic fcDNA treatment (no.=12)	−	−	+
G6 mice: DSS-colitis; colitic fcDNA treatment (no.=12)	+	−	+

G group, *No.* number of animals

formalin overnight and thereafter stored in 70 % of ethanol before embedding in paraffin. Tissue sections of 5 μm were stained with haematoxylin and eosin (H&E) for histological evaluation.

H&E stained slides were digitalized using a high resolution Mirax Desk instrument (Zeiss, Goettingen, Germany) and analyzed with the Mirax TMA Module software (Zeiss).

Histological activity index (HAI) of the colonic samples was calculated using the following criteria: percentage of area involved, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells, as described earlier [20]. The percentage of involved area and crypt loss was scored on a scale of 0–4 (0: normal; 1: <10 %; 2: 10 %; 3: 10 %–50 %; 4: >50 %). Mucosal erosions were scored by 0: if the epithelium was intact; 1: if LP was involved; 2: if ulcerations involved the submucosa; and 3: if ulcerations were transmural. The severity of other parameters was scored on a scale of 0–3 (0: absent; 1: weak; 2: moderate; 3: severe). The number and diameter of LAs/ILFs was also measured. A certified pathologist scored all of the tissue sections (blinded analysis).

Isolation of Lamina Propria Immune Cells

Leukocytes and lymphocytes from the colon were isolated according to the methods described previously [21–23] with some modifications. For the elimination of the epithelial layer, 20 mm-long colon segments were individually transferred from the RPMI-1640 medium into 10 ml of Ca- and Mg-free Hank's Balanced Salt solution (HBSS; H4385, Sigma-Aldrich) containing 5 mM EDTA, and were then incubated at RT for 45 min. in a linear shaker. After removing the supernatant, colon segments were individually placed into RPMI-1640 medium containing 1 mg/ml collagenase (C5138; Collagenase from Clostridium histolyticum Type IV.; 068K8620; Sigma-Aldrich). The tissues were digested and gently shaken for 60 min at 37 °C. After the digestion, suspensions were filtered through a 70 μm cell filter (Cell Strainer, BD Falcon, Soft Flow, Hungary) to remove the cellular debris. Cell suspensions containing mononuclear cells and lymphocytes of the LP were then centrifugated at 1,300 rpm for 10 min. After removing the supernatant, the isolated cells were processed further for RNA isolation.

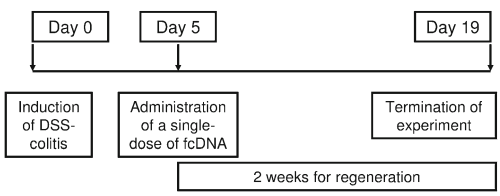

Fig. 1 Schematic view of the experiment

Quantitative Real-Time PCR

Total RNA from the isolated LP cells was extracted with the RNeasy Mini kit (Qiagen, USA) according to the prescription of the manufacturer. After quantitative (Nanodrop) and qualitative analysis (Bioanalyzer Pico 600 chip kit RNA program; RIN>8 in all cases), reverse transcription was performed by using 1 μg of total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA).

For the examination of pattern recognition receptor-related signaling and cytokine profile expression changes in the LP, 16 genes were selected (Table 2). Triplicated quantitative real-time (qRT) PCR was performed using Probes Master and SYBR green (Roche GmbH, Germany). Gene expression levels for each individual sample were normalized to GAPDH expression. Mean relative gene expression was determined and differences were calculated using the 2-ΔC(t) method.

Statistical Analysis

The data were expressed as the mean ± SD. For the statistics, Student's t-test was used. $P<0.05$ was considered as statistically significant.

Ethical Considerations

The experimental protocol was approved by the Ethics Committee of Animal Welfare of the Medical Faculty of Semmelweis University (No.: 22.1/1159/3/2010).

Table 2 The assayed genes and their primer sequences

Short symbol	Gene name	Primer sequences (forward/reverse)
TLR9	Toll-like receptor 9	F_CCGCTTGCTCAACAAGTACA/R_TCGGAAGGTACCAAAGATGC
TIRAP	TIR domain-containing adaptor protein	F_ACAGACAGCCACACAGGATG/R_GTGGCGAGGTAGGTGACATT
TOLLIP	Toll-interacting protein	F_ATGGACCCTTACTGCCGTCT/R_GGCACTGTGCACTGAATGAC
MYD88	Myeloid differentiation primary response gene 88	F_CACTCGCAGTTTGTTGGATG/R_GCGACTTCAGCTCCTTCAGT
IRAK	Interleukin 1 receptor-associated kinase	F_GCATCTCTGCAGGCTCTGA/R_AGAGGAAGGTGGAGGCAGA
TRAF6	TNF-receptor-associated factor 6	F_GGAACCTCTCCAGCTCCTTC/R_CATGGTGTTTGCACTGCTTC
IKB	NF-KB inhibitor	F_TGCACTTGGCTGTGATTCAT/R_GCCTAGGTCATTCTGCAGGT
NFkB	Nuclear factor kappa B	F_CCTGGTGGAGAACTTTGAGC/R_CATGTCCAGGGGTGTGGTA
*TRAM	TRIF-related adapter molecule	F_GCCTTGGGACAAGATCTACG/R_GTGGTACTGCTTGACCACGA
Inflammatory cytokine-associated genes		
IL1A	Interleukin 1-alpha	F_AGCAGCCTTATTTCGGGAGT/R_GTGCAAGTGACTCAGGGTGA
IL1B	Interleukin 1-beta	F_CATGGAATCCGTGTCTTCCT/R_AGCTGTCTGCTCATTCACGA
IL6	Interleukin 6	F_AAATTTCCTCTGGTCTTCTGGA/R_CTCTGAAGGACTCTGGCTTTG
IFNA	Interferon-alpha	F_TCATTCTGCAATGACCTCCA/R_TATGTCCTCACAGCCAGCAG
IFNB	Interferon-beta	F_CCCTATGGAGATGACGGAGA/R_GTCTCATTCCACCCAGTGCT
IL18	Interleukin 18	F_TTGCTTGCCAAAAGGAAGAT/R_ACAAACCCTCCCCACCTAAC
IL10	Interleukin 10	F_CCAGGGAGATCCTTTGATGA/R_AACTGGCCACAGTTTTCAGG

Pattern recognition receptor signaling-associated genes (Toll-like receptor 9—Myd88 dependent; *—Myd88 independent pathways)

Results

Macroscopic Changes

In the case of normal, non-treated, control animals (Group 1), body weight increased according to normal development. The changes of colon length were not significant.

In respect to mice with DSS-colitis (Group 2, 4 and 6), the body weight decreased significantly at day 5. The non-fcDNA-treated animals (Group 2) demonstrated no body weight increase by day 19, while the fcDNA-treated ones (Group 4 and 6) gained significant weight, as compared to the results of day 5. Furthermore, the body weight increase in Group 6 tended to be slightly higher than that witnessed in Group 4.

In cases of non-colitic, normal, fcDNA-treated animals (Group 3 and 5), the increase of body weight was significantly higher compared to Group 1 ($p<0.05$). The body weight increase in Group 5 was slightly higher than that in Group 3 (Table 3).

The average colon length was significantly shorter in DSS-colitic, non-fcDNA treated animals (Group 2) when compared to Group 1. When DSS-colitis mice were treated with fcDNA (Group 4 and 6), the colon lengths were longer compared to Group 2. The length difference was significant in respect to Group 2 and 6 ($p<0.05$). In cases of non-colitic, fcDNA-treated mice (Group 3 and 5), an increase in length was observed, and this was significant between Group 1 and Group 5 ($p<0.05$) (Fig. 2a).

Regarding other examined parenchymal organs (stomach, small bowel, kidneys, spleen, lungs, liver and pancreas), no remarkable macroscopic alterations were detected.

Changes of Disease Activity Index

The complex disease activity index (DAI) containing the changes of body weight, the presence of loose stool and fecal blood, indicated significant decrease in DSS-colitic, fcDNA-treated mice (Group 4 and 6) as compared to DSS-colitic, non-fcDNA-treated animals (Group 2) ($p<0.005$). In the C-fcDNA treatment (Group 6), the decrease of DAI was more significant compared to that in the N-fcDNA-treated group (Group 4) (Fig. 2b).

Histological Changes

On day 19, the complex colonic histological activity index (HAI) showed significant decrease in fcDNA-treated DSS-colitic animals (Group 4: 1.972±0.485; Group 6: 1.278±0.408) compared to the fcDNA-non-treated, DSS-colitic animals (Group 2: 3.111±0.288) ($p<0.005$, respectively). In the C-fcDNA treatment (Group 6), the decline of HAI was more significant than in the N-fcDNA-treated group (Group 4) (Fig. 2c). Regarding other parenchymal organs examined, no significant alterations were found. The histological changes in DSS-induced colitis before and after fcDNA administration can be seen in Fig. 3.

In view of the number and diameter of LAs and ILFs, significant rise was found only in respect to C-fcDNA treatment in Group 5 and 6 respectively, as compared to Groups 1 and 2. In Group 1 LAs/ILFs number/cm was 1.2±0.3, while in the C-fcDNA treatment group (Group 5) it became significantly higher (2.6±0.3; $p<0.05$). In Group 2 LAs/ILFs number/cm was 6.7±0.7, while in the case of C-fcDNA treatment (Group 6), it was found to be significantly higher (8.4±0.9; $p<0.05$) (Fig. 4).

Table 3 Body weight data

	Day 0	Day 5	Day 19
G1 mice (absolute control; no-DSS; no-fcDNA treatment)	20.6±0.5	20.7±0.6	22.9±0.4†,‡
G2 mice (DSS-colitic; no-fcDNA treatment)	20.3±0.3	17.6±0.4*	17.5±0.4†
G3 mice (non-colitic; normal fcDNA treatment)	19.9±0.4	20.7±0.8	23.9±0.9†,‡
G4 mice (DSS-colitic; normal fcDNA treatment)	20.4±0.5	18±0.1*	18.2±0.2†,‡
G5 mice (non-colitic; colitic fcDNA treatment)	20.4±0.4	20.9±0.4	24.1±0.8†,‡
G6 mice (DSS-colitic; colitic fcDNA treatment)	20.4±0.5	17.6±0.7*	18.5±0.5†,‡

* represents significant differences between the body weights on day 5 compared to day 0 ($p<0.05$); † day 19 compared to day 0 ($p<0.001$); ‡ day 19 compared to day 5 ($p<0.05$)

Gene Expression Changes

In the case of N-fcDNA treatment (G3 and G5) no significant gene expression alterations were found (data not shown). However, significant gene expression differences were observed according to C-fcDNA treatment. In both C-fcDNA-treated groups (Group 5 and 6), 15 genes tended to be down-regulated. Five of the 15 genes showed significant ($p<0.05$) down-expression in Group 6 compared to Group 4 (N-fcDNA-treated DSS-colitic mice). These five genes included TRAF (fold change in G4 vs. G6=7.22±0.18 vs. 3.91±0.19) and TOLLIP (6.08±0.25 vs. 2.49±0.34) of the TLR9-signaling pathway, and IFNA (8.47±0.1 vs. 4.94±0.2), IFNB (9.46±0.14 vs. 6.91±0.2) and IKB (5.84±0.14 vs. 3.08±0.17) of the cytokine signaling. Two genes, namely IRAK (6.35±0.12 vs. 10.33±0.21) and IL-18 (6.45±0.15 vs. 8.74±0.35) showed significant ($p<0.05$) overexpression in Group 6 (Figs. 5 and 6).

Discussion

In the gastrointestinal tract, the innate immune system plays a key function in recognizing and determining appropriate host responses to luminal antigens and microbial products [24]. TLRs, representing sentinels of the innate immunity are widely expressed on various cell types of the gastrointestinal epithelium and lamina propria [24]. TLR9 is specifically stimulated upon sequence- and methylation-dependent DNA signaling [8]. Self-DNA and oligonucleotides containing unmethylated CpG motifs are also senzed by and activate TLR9 [8]. Modifications in the structure of nucleic acids influence their immunomodulatory capacity [25–27]. Therefore free DNA may contribute to the pathogenesis of both inflammatory and repair conditions. In order to better understand the pathogenesis of IBD, it must be mandatory to explore the complex signaling pathway interactions between the innate and adaptive parts of the immune response.

In experimental models of colitis, the role of TLR activation, antagonism and deletion has already been examined [28]. Activation of certain TLRs, including TLR9, exerts anti-inflammatory effects, particularly when the agonists are administered prior to induction of colitis [29]. To best of our knowledge, the effect of intravenously administered, isolated and purified self-fcDNA has not yet been examined.

FcDNA in blood originates from different sources [6, 30, 31]. Within inflammatory and tumorous conditions, fcDNA is especially derived from apoptotic/necrotic cells localizing the perivascular/perilymphatic area. However, circulating (tumor) cells disintegrating in the blood stream could serve as another source of fcDNA. Furthermore, fcDNA could be released from circulating lymphocytes via an active secretory pathway, and, additionally, contain pathogen (luminal microbiota)-derived nucleotide sequences.

In chronic colonic disorders diagnostic [32–34], prognostic [35], and therapy monitoring [36] roles of fcDNA have already been examined, but little is known about its real biological function. Our experimental study seems to be the first one in which the immunobiological/therapeutic effects of iv. administered self-fcDNA were assayed in DSS-colitic mice.

Our findings demonstrate that iv. administration of fcDNA originating from colitic mice (C-fcDNA) significantly suppressed the clinical activity of DSS-colitis, since all the body weight, stool consistency, degree of intestinal bleeding, and colonic shortening considerably improved. Histopathologic findings (HAI) were in line with the DAI data, since iv. C-fcDNA administration reduced mucosal damage, and thus, ameliorated inflammation.

High levels of proinflammatory cytokines serve as colonic damage factors [37], not only in human IBD patients, but also in animal models of DSS-colitis [38]. In our experiment, the immunobiological explanation of the observed changes according to the effect of fcDNA might be fairly complex. Since the methylation status of fcDNA can be disease- and organ specific [39, 40], and TLR9 is able to bind CpG DNA sequences [8], the use of artificially modified DNA sequences could indicate a possible and reasonable therapeutic alternative. In human ulcerative colitis, there is an ongoing phase III. clinical trial in which the local immunomodulatory effects of a synthetic DNA-based immunomodulatory sequence

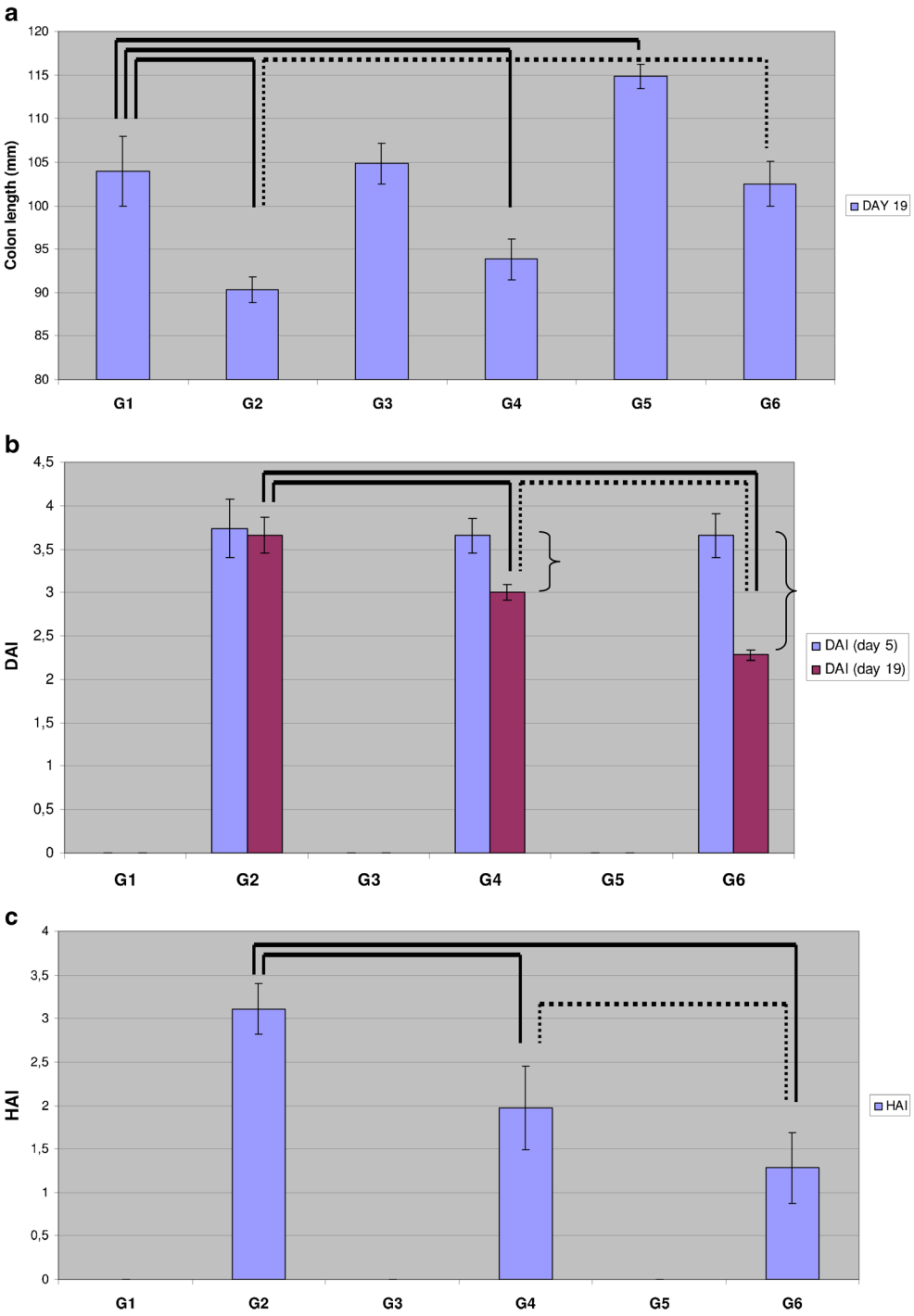

Fig. 2 Changes of colon lenghts, disease activity and histological activity indices. **a** Average colon lengths at day 19. Differences between G1 vs. G2, G1 vs. G4, G1 vs. G5 (indicated with *dark lines*), and G2 vs. G6 (indicated with *dotted lines*) were significant ($p<0.05$); **b** Disease activity indices (DAIs) at day 5 and 19. Differences between DAIs at day 19 of G2 vs. G4, G2 vs. G6 (indicated with *dark lines*), and G4 vs. G6 (indicated with *dotted lines*) were significant ($p<0.005$). Intragroup differences between DAIs in G4 and G6 were also significant (indicated with *square braces*; $p<0.005$); **c** The changes of histological activity indices at day 19. Differences between G2 vs. G4, G2 vs. G6 (indicated with *dark lines*), and G4. vs. G6 (indicated with *dotted lines*) are significant ($p<0.005$)

(DIMS0150) are being studied [17]. The main disadvantage of DIMS0150 is the need of repetitive, colonoscopy-based administration of the treatment. Therefore, in the future a more convenient therapeutic route of administration, i.e. orally or parenterally would be more favorable for the patients.

In our study after fcDNA treatment, the increased density of colonic LAs and ILFs may refer their association with immune cells being able to bind fcDNA via TLR9 [41]. For a better understanding of fcDNA-mediated changes in cytokine-profile and TLR9-signaling pathways, we assayed the related mRNA expression profiles. The effect of C-fcDNA was more remarkable in comparison to those of N-fcDNA. C-fcDNA administration resulted in significant changes of certain TLR9-signaling and proinflammatory cytokine gene expressions. These detected alterations of gene

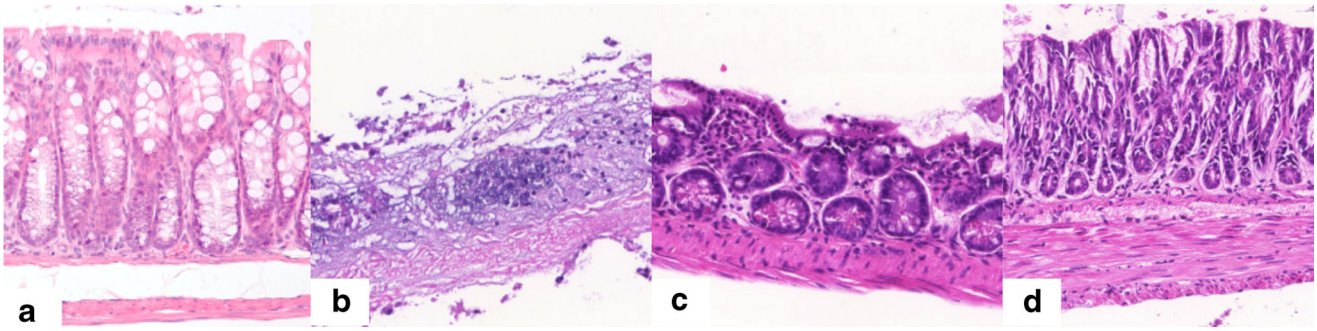

Fig. 3 Histological changes of the colon after iv. fcDNA administration (H&E). **a** Normal, non-treated colon (G1). **b** DSS-induced colitis (G2). **c** DSS-induced colitis treated with normal fcDNA (G4). **d** DSS-induced colitis treated with colitic fcDNA (G6). (170× magnification)

expressions could promote the decrease of the inflammatory activity cascade along with favoring colonic mucosal regeneration.

The DNA-induced modulations of the innate immunity are regulated by TLR-dependent and independent pathways [42]. Upon ligand binding all TLRs (except TLR3) within the so-called canonical pathway recruit directly or indirectly the IL-1R signaling adaptor protein MyD88 via TIR domain-containing adaptor protein (TIRAP) leading to the subsequent recruitment of several members of the IRAK family, and finally, activation of a kinase cascade, culminating in nuclear translocation of NF-kB in a TRAF6-dependent manner [43–46]. MyD88 deficiency in mice leads to susceptibility to experimental colitis [47]. IRAK1, a signaling kinase is considered as an important molecule in early-event signaling, and as a limiting factor for the late-phase one. IRAK1 mainly plays a proinflammatory role in TLR/IL-1R signaling in response to exogenous TLR ligands or endogenous IL-1α/β or IL-18 [44]. DSS-induced colitis is a commonly used CD4+ T-cell-independent murine model resembling human IBD [12]. IRAK1 has been demonstrated to be essential for DSS-induced intestinal inflammation [48]. However, IRAK1 is also involved in the production of the anti-iflammatory IL-10 since in case of IRAK1-deficient mice decreased IL-10 mRNA was measured [48]. Within TLR9 signaling different adaptor use is possible for the diverse CpG motifs of DNA. TRAF6 is

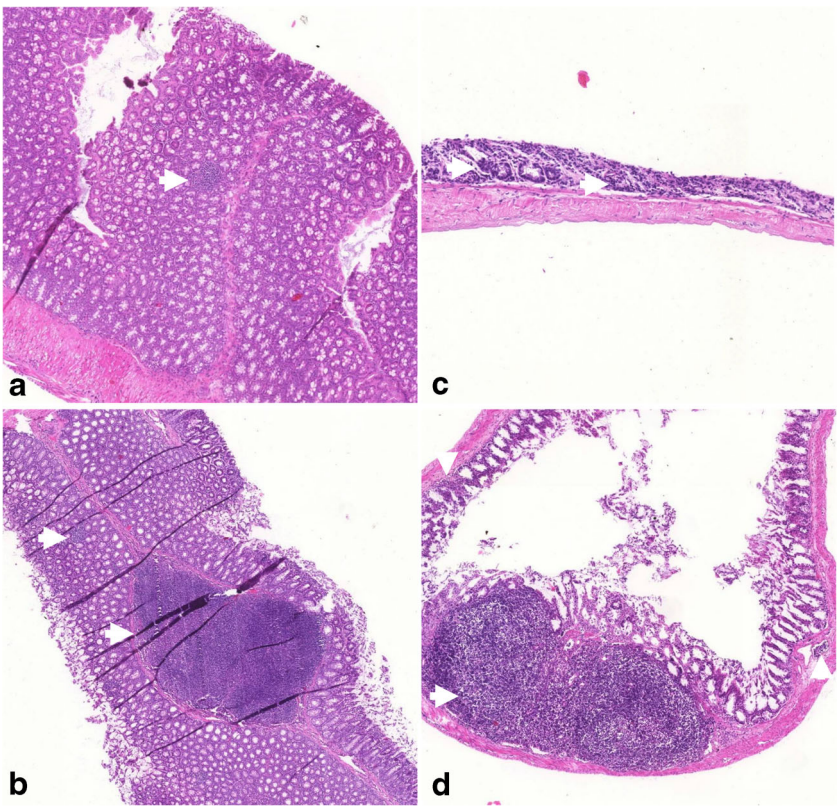

Fig. 4 Isolated lymphoid follicles and lymphoid aggregates in the DSS-induced colitis model after iv. administration of fcDNA (H&E). **a** Normal non-treated control (G1, no-DSS, no fcDNA; 110× magnification). **b** Normal control treated with colitic fcDNA without previous DSS-treatment (G5; 110× magnification). **c** DSS-induced colitis without fcDNA treatment (G2; 110× magnification). **d** DSS-induced colitis treated with colitic fcDNA (G6; 110× magnification). *Arrows* indicate lymphoid follicles and lymphoid aggregates

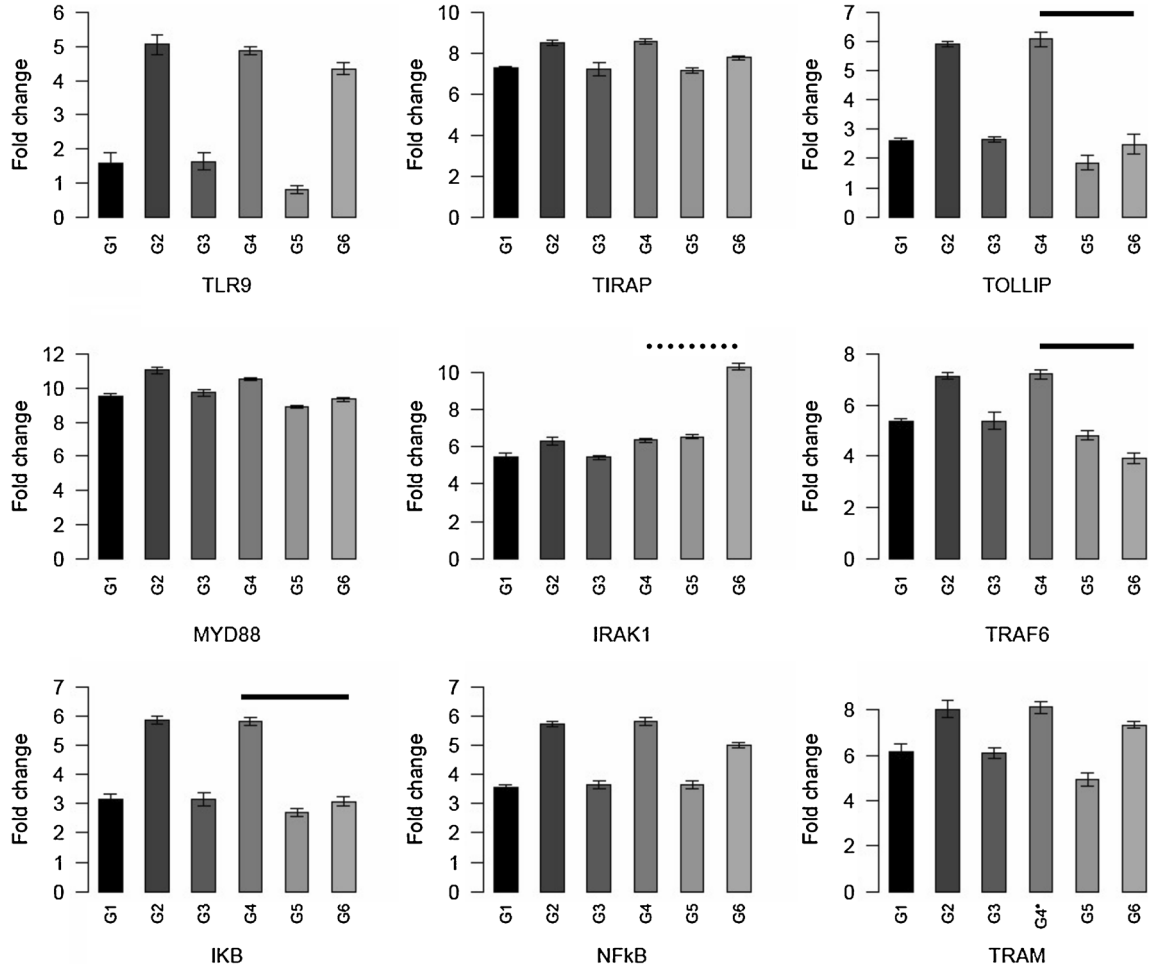

Fig. 5 Foldchange alterations of the assayed genes. Expression changes of the pattern recogntion receptor signaling-associated genes (*dark lines* indicate significant decrease in gene expression regarding G4 vs. G6 groups; *dotted line* indicates significant increase in gene expression regarding G4 vs. G6 groups)

differentially involved in MyD88- and IRAK1-induced activation of NF-κB. One cannot exclude the possibility that the activation of NF-κB in response to IRAK1 overexpression is qualitatively different from the activation induced physiologically in response to TLR/IL-1R stimulation. It is also known that TLR-signaling may be blunted via distinct molecular mechanisms including the expression of the inhibitory molecule TOLLIP [25].

Based on earlier studies, fcDNA bound by TLRs may influence the expression of IDO-1, which hypothetically might be a factor in mediating the beneficial effects of iv. fcDNA administration [15]. Recently it has been found that structurally and functionally distinct types of CpG-DNAs exert different actions via TLR9, favoring either enormous NF-kB activation and immediate induction of proinflammatory cytokines, or provoking the production of IFNA, and thus eliciting reparation activity [25]. Mice lacking IRAK1 failed to produce IFNA in response to DNA, suggesting that the signaling complex of MyD88 with IRAK1 has an essential role in significant TLR9-mediated type I/II IFN production [49–51]. However, type-I IFN secretion can be induced by DNA via TLR9-independent pathway, suggesting that this innate immune activation may also be independent of CpG motifs [25].

Activation of NF-kB is initiated by the signal-induced degradation of IKB proteins [25]. NF-kB activated upon various cytokine receptors and pattern recognition receptors is a key master transcriptional regulator that controls the expression of proinflammatory mediators. In addition, ablation of enterocyte-specific NF-kB signaling provokes massive intestinal inflammation [52]. The dynamics of IKB degradation and subsequent NF-κB transcriptional activity in response to diverse ways of stimulation may partly be involved in this phenomenon [53].

The polarity of intestinal epithelial cells (IECs) crucially affects the response of TLR9 to DNA ligation [54]. Hence, stimulation of apical TLR9 results in accumulation of NF-kB inhibitors, thus blocking its activation, and in suppression of inflammation, partly due to the induction of type-I IFNs, whereas activation of basolateral TLR9 induces NF-kB and

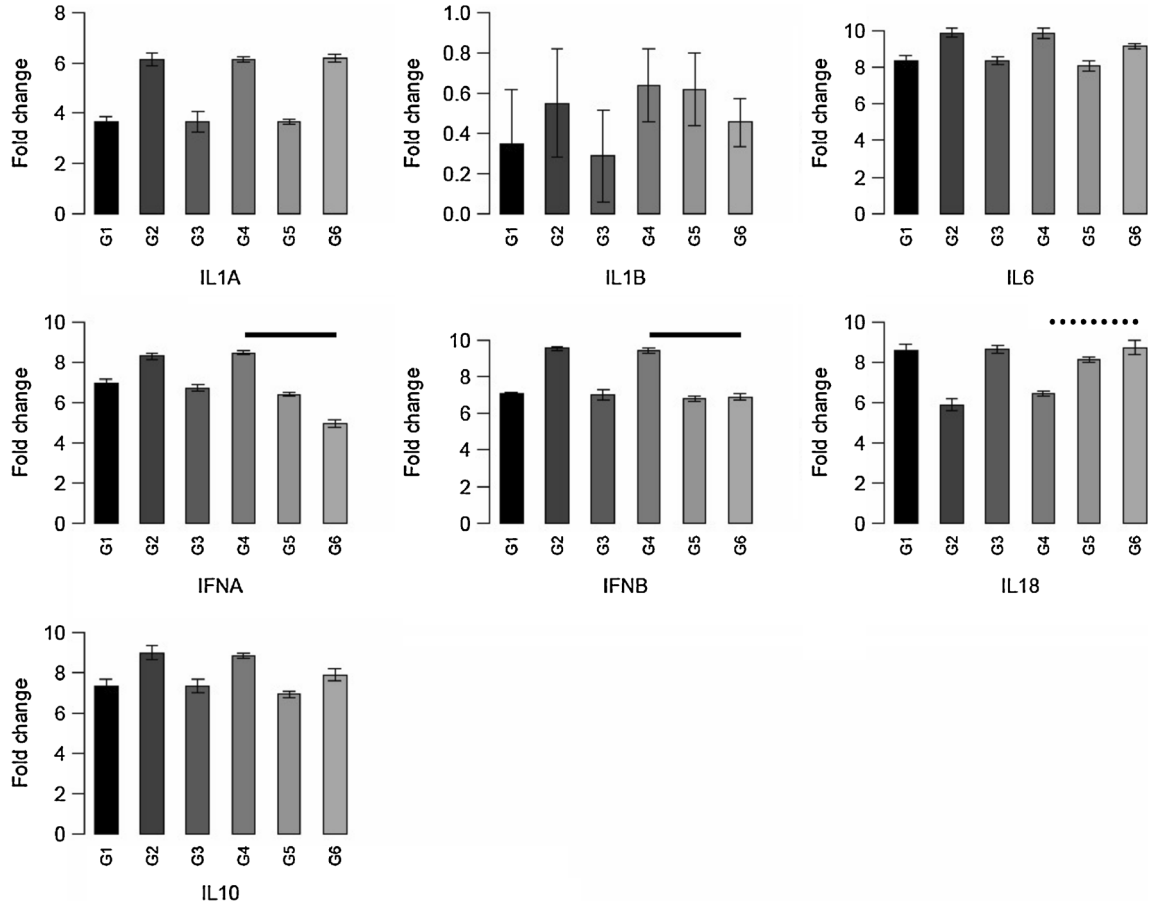

Fig. 6 Foldchange alterations of the assayed genes. Expression changes of the inflammatory cytokine-related genes (*dark lines* indicate significant decrease in gene expression regarding G4 vs. G6 groups; *dotted line* indicates significant increase in gene expression regarding G4 vs. G6 groups)

proinflammatory responses. In tissue regeneration type-I IFNs are considered as protective cytokines against colonic inflammation. Under severe inflammatory conditions, however IECs may lose polarity [55].

Inflammasome-related IL-18 signaling via MyD88 exerts a dual role in intestinal homeostasis and colitis, since it is required for DSS-induced colitis, as well as for compensatory proliferation of IECs, and tissue repair [42]. Recent studies in DSS-induce colitis suggest that in IECs the IL-18/MyD88 signaling axis may be responsible for activating still unknown mechanisms inducing efficient mucosal repair [56]. Loss of the innate immune effector molecules can deeply alter tissue regeneration, and promote excessive mucosal inflammation [57]. However, the exact mechanism of IL-18 regarding its protective, healing effect on colonic mucosa has not yet been clarified.

It would be important to ascertain the exact cellular origin of fcDNA, essentially, the originating tissues. The colon-specificity of circulating methylated-septin-9 DNA sequences has already been proven [58]. We hypothesize that in our experiment at least a fraction of pooled fcDNAs could originate from the inflamed colonic mucosa. To solve this unexplained question, a comparative analysis, i.e. detection of the hypothetic organ-specific methylation pattern of fcDNA could be of importance.

Our present experiment indicates that within inflammatory circumstances systemic administration of colitis-derived fcDNA can reduce the clinical and histopathologic severity of DSS-induced murine colitis, possibly through altering pro-inflammatory cytokine expression and TLR9-related signaling. Presumably due to induction of severe colitis, the subsequent presence of a marked inflammatory environment may result in fcDNA with a potential to promote the downregulation of inflammation and improve tissue regeneration. A balance between proinflammatory and repair signals of the immune system is essential for maintaining intestinal homeostasis. Elucidating mechanisms of innate immune alterations by nucleic acids may have provide further insight into the etiology of inflammatory bowel diseases and develop the basis of novel, more efficient nucleic acid-based immunotherapies.

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