Regulatory role of suppressive motifs from commensal DNA

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The microbiota contributes to the induction of both effector and regulatory responses in the gastrointestinal (GI) tract. However, the mechanisms controlling these distinct properties remain poorly understood. We previously showed that commensal DNA promotes intestinal immunity. Here, we find that the capacity of bacterial DNA to stimulate immune responses is species specific and correlated with the frequency of motifs known to exert immunosuppressive function. In particular, we show that the DNA of *Lactobacillus* species, including various probiotics, is enriched in suppressive motifs able to inhibit *lamina propria* dendritic cell activation. In addition, immunosuppressive oligonucleotides sustain T_{reg} cell conversion during inflammation and limit pathogen-induced immunopathology and colitis. Altogether, our findings identify DNA-suppressive motifs as a molecular ligand expressed by commensals and support the idea that a balance between stimulatory and regulatory DNA motifs contributes to the induction of controlled immune responses in the GI tract and gut immune homeostasis. Further, our findings suggest that the endogenous regulatory capacity of DNA motifs enriched in some commensal bacteria could be exploited for therapeutic purposes.

INTRODUCTION

The human intestine harbors 1,000 trillion microbes composed of an estimated 4,000 strains.[1] The commensal flora has a fundamental role in various aspects of host physiology, including tissue development, metabolism, and immunity. This symbiotic relationship is controlled, at least in part, by interaction of host cells with commensal-derived microbe-associated molecular patterns. Paradoxically, commensal and pathogenic microbes interact with the host immune system through similar conserved ligands such as the ones signaling through the Toll-like family of receptors (TLR).[2] TLR signaling in the intestinal epithelial compartment is critically involved in the maintenance of intestinal homeostasis and tissue repair.[3] Further, these signals also positively regulate the sampling of luminal contents by dendritic cells (DCs) from the underlying *lamina propria* (LP) compartment and can mediate tolerance to food antigens.[4,5]

TLR9 recognizes unmethylated cytosine phosphate guanosine (CpG) dinucleotides, which are abundant in prokaryotic DNA found in intestinal flora. In mice, we found that constitutive gut flora DNA sensing in the gastrointestinal (GI) tract can act as an immunological adjuvant and critically controls the balance between regulatory (T_{reg}) and effector T cells.[6] Although commensal communities can control immune system development and function, individual bacteria can have highly distinct properties. For instance, some bacteria, referred to as probiotics, can promote regulatory responses in the GI tract, while others, referred to as pathobionts, can, in some circumstances, contribute to pathology.[7,8] Some of these distinct properties have been linked to the expression of defined ligands or expression of factors associated with enhanced adhesion with epithelial cells. However, the molecular basis of the functional disparities between commensal species remains poorly understood.

DNA from pathogenic bacteria are not all equal in their capacity to stimulate TLR9 and do so with various levels of efficiency that correlates with their frequency of CpG dinucleotides.[9,10] Thus, it could be proposed that increased CpG motifs in the

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DNA of defined commensal bacteria may contribute to their enhanced inflammatory properties. An alternative possibility is that the DNA of a subset of commensal bacteria may be more enriched in what have been referred to as suppressive motifs. These motifs are poly guanosine- or [GC]-rich sequences, are enriched in the telomere region of mammalian DNA, and have been shown to selectively block immune activation induced by CpG oligonucleotides (ODN).[11–14] Although their presence has not been reported in bacteria, we hypothesized that increased frequency of these motifs at the DNA level may potentially contribute to the functional specificity of commensal bacteria on host immune responses.

Our present results reveal that the capacity of bacterial DNA to stimulate immune responses is bacteria specific. Strikingly, suppressive motifs, enriched in the DNA of the probiotic of the *Lactobacillus* family, potently prevent DC activation and maintain T_{reg}-cell conversion in the face of inflammation. Additionally, treatment of mice with suppressive ODN (sup-ODN) mitigates tissue damage and inflammation associated with oral *Toxoplasma gondii* infection and dextran sulfate sodium (DSS)-induced colitis. Altogether, our work provides evidence that DNA motifs in the microbiota may contribute to the hierarchy of commensal-derived signals and maintenance of gut immune homeostasis. Further, our findings suggest that the endogenous regulatory capacity of DNA motifs enriched in specific commensal bacteria could be harnessed for therapeutic purposes.

RESULTS

DNA from distinct commensal bacteria differentially stimulates intestinal immune responses

To assess whether functional differences in response to commensals is apparent at their DNA level, we evaluated the proinflammatory capacity of the DNA from two prototypic commensal bacteria with known divergent effects on the host immune system. In particular, we used DNA isolated from the probiotic *Lactobacillus paracasei* and a commensal with pathogenic potential *Escherichia coli*.[7] To ensure the predominance of these signals, we placed 3-week-old mice on a cocktail of antibiotics (ATBs).[3] After 6 weeks of the ATB course, mice were treated orally once a week with CpG ODN or highly purified genomic DNA from *E. coli* or *L. paracasei*. Two weeks later, mice were orally infected with the GI parasite *Encephalitozoon cuniculi* that induces strong T-helper (Th) type 17 and Th1 responses, the latter required for host protection.[6,15] As previously shown, reduction of the gut flora significantly impaired both local and systemic interferon (IFN)-γ and interleukin (IL)-17 responses and compromised parasite control (**Figure 1a,b**).[6] In a comparable manner to CpG ODN treatment, *E. coli* DNA significantly restored effector responses and parasite control (**Figure 1a,b**). By contrast, DNA from *L. paracasei* failed to rescue either IFN-γ or IL-17 responses and consequently had no effect on the parasite burden compared with ATB treatment alone (**Figure 1a,b**). Thus, at an equivalent dose, DNA from distinct bacterial species has a differential capacity to stimulate immune responses.

E. coli and *L. paracasei* DNA differentially activate LP DC (LpDC) and influence T_{reg}-cell induction

The intestinal mucosa contains a large number of antigen-presenting cells that have an important role in shaping adaptive immunity in response to intestinal environmental cues.[16] We therefore assessed whether our *in vivo* observations could correlate with differential effects of *L. paracasei* and *E. coli* DNA on gut resident DCs by performing *in vitro* experiments using LpDC purified from naive mice. The small intestine LP contains both resident and migratory DCs, the latter tending to express high levels of CD103.[17,18] Upon stimulation with CpG ODN, both LpDC subsets produced tumor necrosis factor (TNF)-α and IL-12/23p40 (**Figure 2a**). High levels of IL-6 were also measured in the supernatant of CpG ODN-stimulated cultures of purified $CD11c^{hi}$ $MHCII^{+}$ (major histocompatibility complex) LpDCs (**Figure 2b**). Similarly, stimulation with *E. coli* DNA induced TNF-α, IL-12/23p40, and IL-6 secretion by LpDCs albeit to a lower degree than highly purified CpG ODN (**Figure 2a,b**). By contrast, a similar dose of *L. paracasei* DNA was not able to trigger proinflammatory cytokine production by LpDCs (**Figure 2a,b**).

Another property of intestinal LpDCs is their ability to enhance transforming growth factor (TGF)-β-mediated T_{reg}-cell conversion.[19–22] We previously showed that gut flora DNA impaired T_{reg}-cell conversion via its capacity to activate DCs.[6] We utilized this feature as a functional read out for the *ex vivo* effect of the DNA from *E. coli* and *L. paracasei*. Purified LpDCs and naive $CD4^{+}Foxp3^{-}$ T cells were co-cultured in T_{reg}-cell–polarizing conditions, in the presence or absence of CpG ODN or the two types of bacterial DNA. To specifically assess the effect of DNA on DCs, we utilized T cells from *Tlr9*$^{-/-}$ mice. As we previously demonstrated, stimulation with CpG ODN led to a dramatic decrease in the frequency of $Foxp3^{+}$ cells, whereas $\alpha_4\beta_7$ expression, which mediates migration of cells to the gut, was maintained on Foxp3-negative cells (**Figure 2c**). Similarly, the proportion of $Foxp3^{+}$ cells was strongly reduced in a TLR9-dependent manner in the presence of *E. coli*-derived DNA (**Figure 2c** and **Supplementary Figure S1** online). By contrast, a similar amount of *L. paracasei* DNA had no effect on the induction of T_{reg} cells (**Figure 2c**). To assess whether such an effect was due to a complete inability of the probiotic DNA to activate LpDC, we performed a similar assay in the presence of increasing doses of DNA. Using this approach, we found that *L. paracasei* DNA was not inert but 100 times less potent at inhibiting T_{reg}-cell induction compared with the DNA of *E. coli* (**Figure 2d**). Thus, the *L. paracasei* DNA has the capacity to stimulate antigen-presenting cells but does so in a less efficient manner than the DNA from *E. coli*.

L. paracasei genome is enriched in immunosuppressive motifs

Previous studies have shown that, *in vitro*, DNA from distinct pathogenic bacteria stimulate TLR9 with varying levels of efficiency that correlate with the frequency of unmethylated CpG dinucleotides in their DNA.[9,10] However, assessment of the frequency of prototypic stimulatory motifs AACGAT

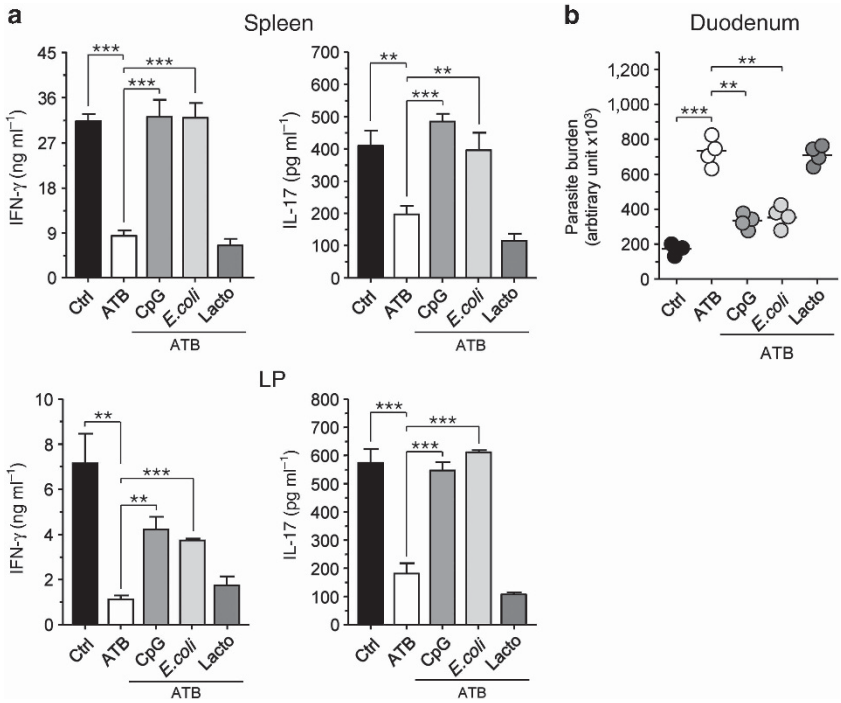


Figure 1 *Lactobacillus paracasei* (*Lacto*) and *Escherichia coli* (*E. coli*) DNA differ in their capacity to restore intestinal effector immune responses in antibiotic (ATB)-treated mice. A cocktail of ATBs in the drinking water was given to 3-week-old mice. Six weeks after the start of the ATB regimen, mice received oral weekly treatments consisting of phosphate-buffered saline alone (ATB) or containing 100 μg of cytosine phosphate guanosine oligonucleotides (CpG ODN) 1826, 500 μg of DNA from *L. paracasei* or 500 μg of DNA from *E. coli*. Control (Ctrl) mice received regular water and no treatment. All mice were orally infected with *Encephalitozoon cuniculi* 8 weeks after the beginning of the ATB course. (**a**) ELISA of interferon (IFN)-γ and interleukin (IL)-17 in supernatants of bulk leukocyte preparations from spleen and small intestine *lamina propria* (LP) of 11 days post-infected mice, restimulated with *E. cuniculi*-infected bone marrow-derived dendritic cells for 72 h. Histograms represent the mean cytokine concentration of triplicate wells ± s.d. (**$P<0.01$; ***$P<0.001$). (**b**) Parasite burden was evaluated in duodenum 11 days after infection by quantitative real-time PCR. Each dot represents one mouse and each bar represents the mean of three of four mice analyzed (**$P<0.01$; ***$P<0.001$). All data shown are representative of two independent experiments with similar results.

and ATCGAT in the sequenced genome of 27 strains of *E. coli* and 28 *Lactobacillus* species showed similar relative expression between *E. coli* and bacteria of the *Lactobacillus* genus (see **Supplementary Tables 1** and **2** and **Supplementary Figure S2a** online). An alternative but not exclusive explanation for our aforementioned results is that DNA from *L. paracasei* would have increased regulatory capacity. We next compared the frequency of suppressive motifs.[23,24] Notably, we found that the suppressive sequences, TTAGGG and TCAAGCTTGA, were enriched in the DNA of all *Lactobacillus* species compared with *E. coli*, including those with reported probiotic activities (**Figure 3a,b** and see **Supplementary Figure S2b,c** online). Notably, reduced levels of suppressive motifs do not correlate with the pathogenicity of *E. coli* strains (**Figure 3a,b** and see **Supplementary Figure S2b,c** online). These findings suggest that a shift in the ratio of regulatory vs. stimulatory motifs might contribute to the differential effect of commensal DNA on immune responses.

Sup-ODN reduce inflammatory cytokine production by gut antigen-presenting cells

Previous work has established that sup-ODN could inhibit the production of proinflammatory cytokines by CpG ODN-stimulated splenic cells.[13,14,25] To address the possibility that suppressive motifs could interfere with the adjuvant property of CpG ODN at mucosal sites, we synthesized two ODNs containing repeats of the suppressive sequences TTAGG or TCAAGCTTGA: A151 (sup-ODN1) and H154 (sup-ODN2), respectively.[23,24] Addition of sup-ODN1 to the CpG ODN-treated cultures of DC purified from the small intestine LP significantly blocked their production of TNF-α, IL-12/23p40, and IL-6 (**Figure 3c,d**). Notably, co-treatment with control scrambled ODN (ns-ODN) that does not contain stimulatory or suppressive motifs had no effect on the secretion of TNF-α, IL-12/23p40, and IL-6 by LpDCs following stimulation with CpG ODN (**Figure 3c,d**). Based on the profound inhibitory effect of sup-ODN on gut DC inflammatory cytokine production, we next assessed the physiological consequences of these motifs on gut immune responses and pathology.

Sup-ODN inhibit the adjuvant effect of CpG on intestinal immune responses

We first addressed the possibility that suppressive motifs could interfere with CpG adjuvant property at mucosal sites. A group of ATB-treated mice received CpG ODN alone or in combination with sup-ODN1 or sup-ODN2 starting a week before

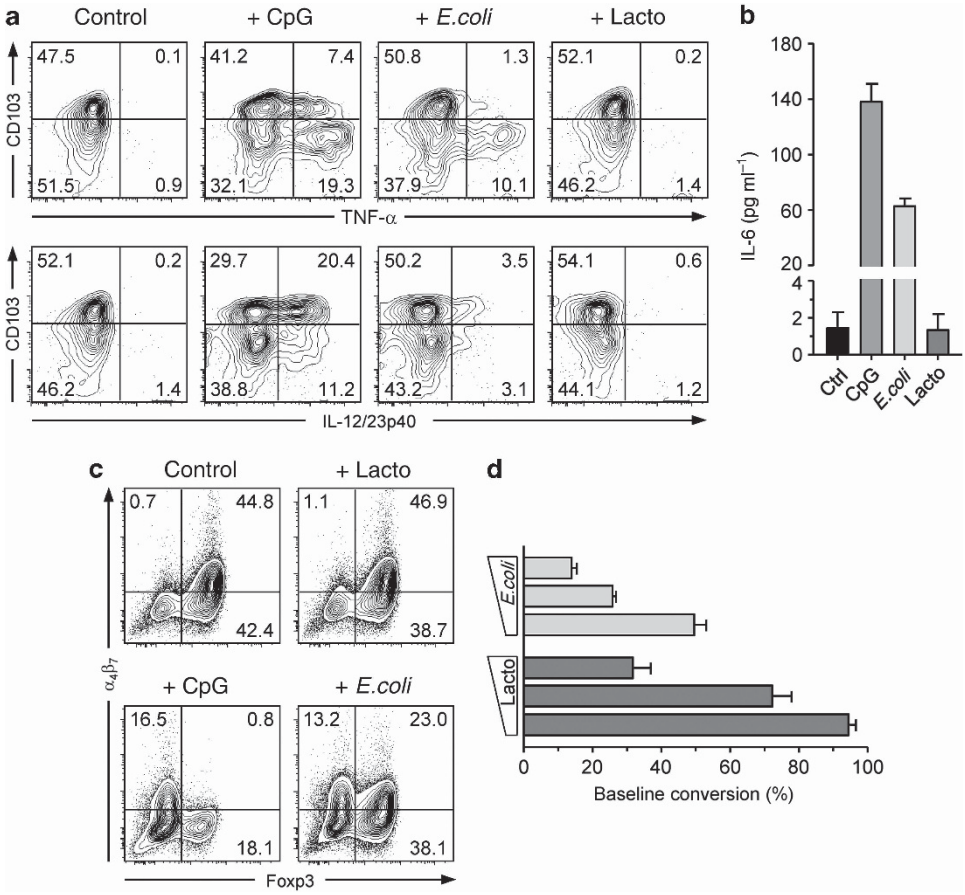


Figure 2 DNA from *Lactobacillus paracasei* (*Lacto*) and *Escherichia coli* (*E. coli*) exert distinct modulatory effects on cytokine production by *lamina propria* (LP) dendritic cells (LpDC) and T_{reg} conversion *in vitro*. (**a**, **b**) *E. coli* DNA is a potent inducer of proinflammatory cytokine secretion by LpDCs. (**a**) LP leukocytes prepared from wild-type naive mice were stimulated for 5 h in the presence or absence of cytosine phosphate guanosine oligonucleotides (CpG ODN) 1555 (1 μg ml^{-1}), *L. paracasei* DNA (0.1 μg ml^{-1}) or *E. coli* DNA (0.1 μg ml^{-1}) in complete media containing brefeldin A. Dot plots represent tumor necrosis factor (TNF)-α or interleukin (IL)-12/23p40 vs. CD103 expression on viable CD11c^{hi} MHCII^{+} (major histocompatibility complex) DCs analyzed by flow cytometry after intracellular cytokine staining. Numbers in quadrant refer to the percentage of each subset. (**b**) Fluorescence-activated cell sorter (FACS)-purified CD11c^{hi} MHCII^{+} LpDCs were stimulated for 18 h in the presence or absence of CpG ODN 1555 (1 μg ml^{-1}), *L. paracasei* DNA (1 μg ml^{-1}), or *E. coli* DNA (1 μg ml^{-1}) in complete media containing 40 ng ml^{-1} of GM-CSF (granulocyte macrophage colony-stimulating factor). IL-6 was then measured in the culture supernatant by enzyme-linked immunosorbent assay. Histograms represent the mean cytokine concentration of triplicate wells ± s.d. (**c**, **d**) FACS-sorted CD4^{+} CD25^{-} CD44^{lo} Foxp3^{-} T cells isolated from Foxp3^{eGFP} *Tlr9* $^{-/-}$ naive mice were cultured in T_{reg} cell–polarizing conditions with CD11c^{hi} MHCII^{+} wild-type LpDCs in the presence of the indicated treatments. As shown in (**c**), dot plots illustrate $\alpha_4\beta_7$ vs. Foxp3 expression on viable TCR-β^{+} (T-cell receptor-β^{+}) CD4^{+} T cells after 5 days of culture in the presence or absence of CpG ODN 1555 (1 μM), *E. coli* DNA (0.1 μg ml^{-1}) or *L. paracasei* (0.1 μg ml^{-1}). As shown in (**d**), *E. coli* DNA or *L. paracasei* DNA was added in the culture at starting concentrations of 10 μg ml^{-1}. Two subsequent 10-fold dilutions of bacterial DNA were tested (white edge). Results were normalized to conditions plated in the absence of any bacterial DNA (100% conversion). Cross bars indicate the highs and lows of duplicate cultures. All data shown in this figure are representative of at least three independent experiments with similar results.

E. cuniculi infection. Strikingly, sup-ODN1 or sup-ODN2 treatment interfered with the protective role of CpG ODN and limited parasite control (**Figure 4a,b**). Thus, suppressive motifs naturally enriched in some commensal species can limit the adjuvant effect of CpG on intestinal immune responses.

We and others previously showed that inflammation interferes with T_{reg}-cell conversion *in vitro* and *in vivo*.[26,27] To evaluate whether sup-ODN could prevent such an outcome, we first tested its potential effects *in vitro*. To this end, CpG ODN was added with or without sup-ODN1 to T_{reg}-cell–polarizing co-cultures of LpDCs and naive CD4^{+}Foxp3^{-} T cells. After 5 days, the percentages of CD4^{+} cells expressing Foxp3 were measured by flow cytometry. As expected, the addition of CpG ODN strongly suppressed T_{reg}-cell conversion (**Figure 5a,b**). Strikingly, sup-ODN1 interfered with the inhibitory effect of CpG ODN, whereas ns-ODN, devoid of stimulatory or suppressive motifs, had no effect (**Figure 5a,b**).

Treatment with sup-ODN sustains T_{reg}-cell conversion and ameliorates the intestinal immunopathology during *T. gondii* infection and DSS-induced colitis

We next assessed the effect of sup-ODN on T_{reg}-cell induction during an inflammatory response *in vivo*. To this end, we used the well-characterized model of acute infection with the intracellular protozoan parasite *T. gondii*. Following oral infection,

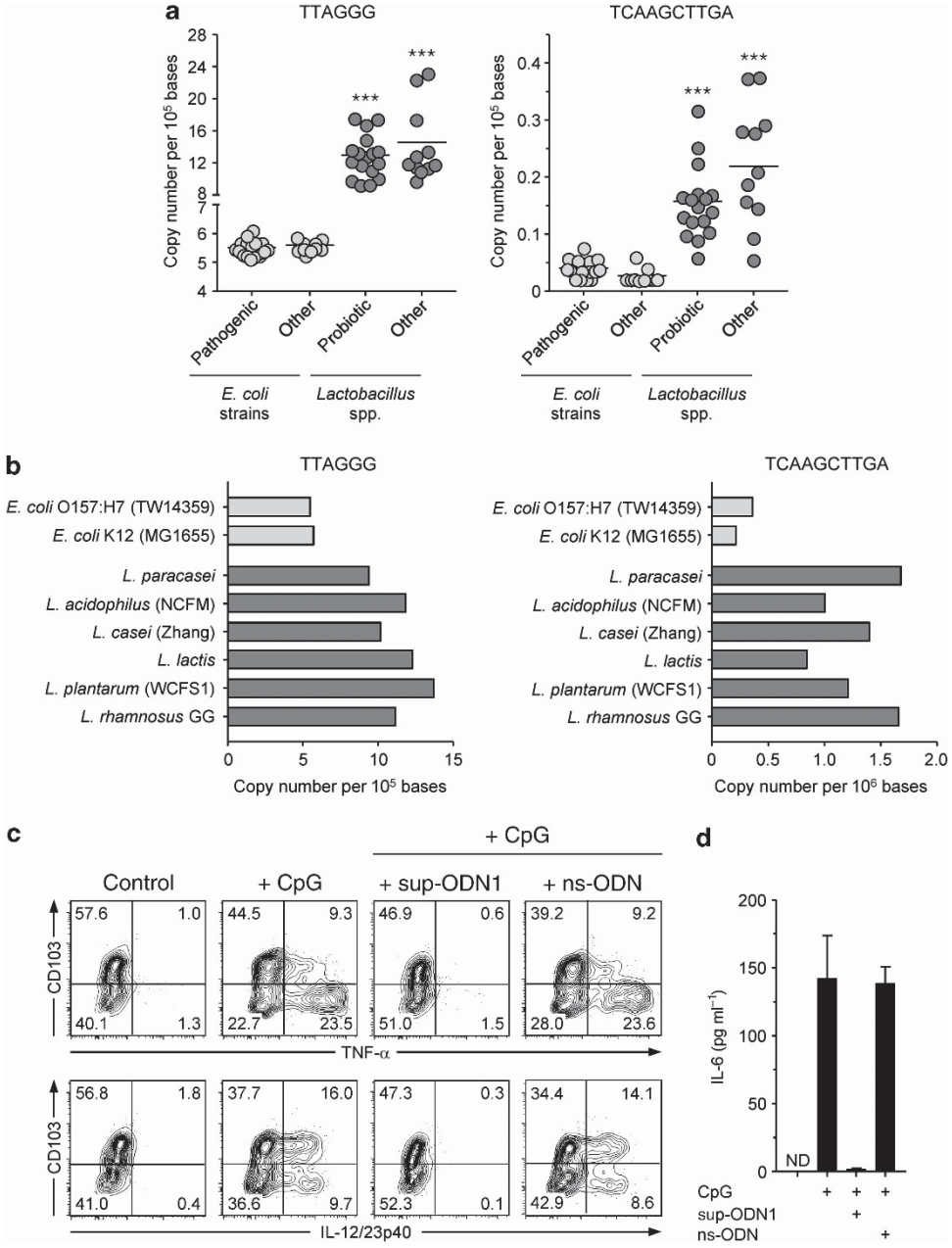


Figure 3 *Lactobacillus paracasei* DNA is enriched in suppressive motifs able to inhibit intestinal *lamina propria* (LP) dendritic cells (LpDC) activation. (**a**, **b**) The sequenced genomes of 27 strains of *E. coli* and 28 species of *Lactobacillus* bacteria were analyzed for the presence of two suppressive motifs: TTAGG and TCAAGCTTGA. Each dot or graph bar represents the number of suppressive motifs per 10^5 or 10^6 bases for each bacterium. (**a**) DNA from *Lactobacillus* spp. bacteria contains higher frequencies of immunosuppressive motifs than DNA from different *E. coli* strains. ***$P<0.001$ (compared with pathogenic or other *E. coli* strains). (**b**) Number of TTAGG and TCAAGCTTGA motifs in two *E. coli* strains and six probiotic *Lactobacillus* bacteria, including *L. paracasei*. (**c** and **d**) Suppressive oligonucleotide 1 (sup-ODN1) inhibits LpDC cytokine secretion induced by cytosine phosphate guanosine (CpG) stimulation. (**c**) LP leukocytes prepared from wild-type naive mice were stimulated for 5 h in the presence or absence of CpG ODN 1555 (1 μM) alone or in combination with sup-ODN1 (1 μM) or control ODN (ns-ODN; 1 μM), in complete media containing brefeldin A. Dot plots represent tumor necrosis factor (TNF)-α or interleukin (IL)-12/23p40 vs. CD103 expression on viable $CD11c^{hi}$ $MHCII^+$ (major histocompatibility complex) DCs analyzed by flow cytometry after intracellular cytokine staining. Numbers in quadrant refer to the percentage of each subset. (**d**) FACS (fluorescence-activated cell sorter)-purified $CD11c^{hi}$ $MHCII^+$ LpDCs were stimulated for 18 h in the presence or absence of CpG ODN 1555 (1 μM) alone or in combination with sup-ODN1 (1 μM) or ns-ODN (1 μM) in complete media containing 40 ng ml^{-1} of GM-CSF (granulocyte macrophages colony-stimulating factor). IL-6 was then measured in the culture supernatant by enzyme-linked immunosorbent assay. Histograms represent the mean cytokine concentration of triplicate wells ± s.d. ND, not detected.

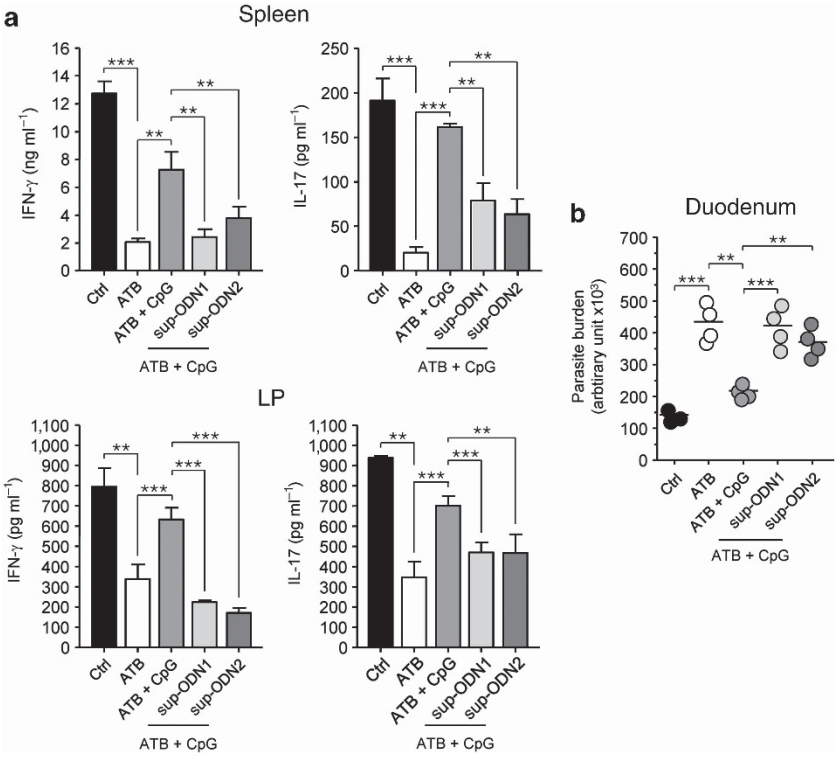


Figure 4 Suppressive oligonucleotides (sup-ODN) limit the adjuvant effect of cytosine phosphate guanosine (CpG) oligonucleotides on intestinal immune responses in antibiotic (ATB)-treated mice. A cocktail of ATBs in the drinking water was given to 3-week-old mice. Seven weeks after the beginning of the ATB regimen, all mice were infected orally with *Encephalitozoon cuniculi*. In addition, some mice were treated with phosphate-buffered saline alone (ATB) or containing CpG ODN 1826 (25 μg), sup-ODN1 (25 μg), and/or sup-ODN2 (25 μg), by gavage on days −6 and −3 before the infection and on days 1, 4, and 8 after the infection. Control mice (Ctrl) received regular water and no treatment. At day 11 post-infection, mice were euthanized and immune responses and parasite loads were analyzed. (**a**) Levels of interferon (IFN)-γ and interleukin (IL)-17 were measured by enzyme-linked immunosorbent assay in supernatants of bulk leukocyte preparations from infected spleen and small intestine *lamina propria* (LP) restimulated with *E. cuniculi*-infected bone marrow-derived dendritic cells for 72 h. Histograms represent the mean cytokine concentration of triplicate wells ± s.d. (***P*<0.01; ****P*<0.001). Data shown are representative of two independent experiments with similar results. (**b**) Parasite burden was evaluated in duodenum by quantitative real-time PCR. Each dot represents one mouse and each bar represents the mean of three of four mice analyzed (***P*<0.01; ****P*<0.001). All data shown are representative of two independent experiments with similar results.

T. gondii induces a strong inflammatory response in the GI tract, and control of the parasite and development of immunopathology are under the control of IFN-γ–producing $CD4^+$ T cells and IL-22, respectively.[28,29] Congenic $CD4^+Foxp3^-$ T cells from OT-II Tg×$Foxp3^{eGFP}$ mice were adoptively transferred into recipients that were infected, or not, with 10 cysts of the type II *T. gondii* strain ME-49. Recipient mice were then fed ovalbumin antigen dissolved in drinking water and treated every other day with sup-ODN1. As we have previously shown, the frequency of cells expressing Foxp3 *de novo* was dramatically reduced in the LP after infection (**Figure 6a**).[27] By contrast, infected recipient mice treated with sup-ODN1 sustained T_{reg}-cell conversion to a greater extent compared with untreated infected animals (**Figure 6a**). Collectively, these data demonstrate that sup-ODNs are sufficient to maintain a cardinal feature of gut physiology, T_{reg}-cell conversion, during acute infection.

Sup-ODNs have been shown to suppress inflammation in several experimental settings.[23,30–32] Based on the role of sup-ODN in limiting LpDC activation and sustaining T_{reg}-cell induction, we postulated that these suppressive motifs could represent a therapeutic approach to limit mucosal inflammation. C57BL/6 mice were infected orally with *T. gondii* and treated orally or not with sup-ODN1. At day 9 post-infection, pathology and antigen-specific cytokine responses from various sites were evaluated. As previously observed,[33,34] *T. gondii* oral infection induced the development of robust Th1 and proinflammatory cytokine responses (IFN-γ, TNF-α, IL-6, and IL-22), in the small intestine LP, mesenteric lymph nodes and spleen (**Figure 6b**). As a byproduct of immune responses, mice developed a severe form of intestinal inflammation characterized by loss of epithelial architecture in the ileum, shortened villi, massive influx of inflammatory cells, and scattered patches of necrosis, as well as severe liver damage, indicated by increased concentrations of serum alanine aminotransferase (**Figure 6c,d**). Strikingly, sup-ODN1 significantly reduced the Th1 response to *T. gondii* as well as the production of cytokines involved in *T. gondii* pathogenesis such as TNF-α, IL-6 and IL-22, in all the compartments analyzed (**Figure 6b**). By contrast, the immunoregulatory cytokine IL-10 required to limit tissue damage in this model[35] was selectively increased in the LP of sup-ODN1–treated animals compared

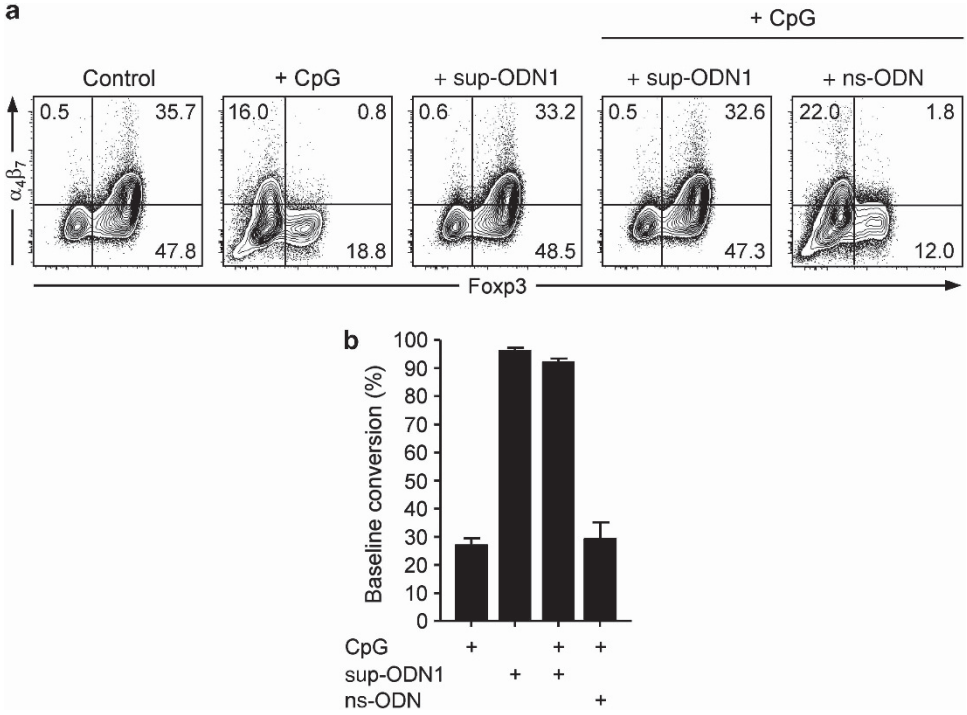


Figure 5 Suppressive oligonucleotides (sup-ODN) reverses the inhibition of T_{reg} cell conversion by cytosine phosphate guanosine (CpG) oligonucleotides *in vitro*. FACS (fluorescence-activated cell sorter)-sorted $CD4^+$ $CD25^-$ $CD44^{lo}$ $Foxp3^-$ T cells isolated from $Foxp3^{eGFP}$ *Tlr9* $^{-/-}$ naive mice were cultured in T_{reg} cell–polarizing conditions with $CD11c^{hi}$ $MHCII^+$ (major histocompatibility complex) wild-type LpDCs (*lamina propria* dendritic cells). The following treatments were added alone or in combination: CpG ODN 1555 (1 μM), sup-ODN1 (1 μM), and control ODN (ns-ODN; 1 μM). As shown in (**a**), dot plots illustrate $\alpha_4\beta_7$ vs. Foxp3 expression on viable TCR-β^+ (T-cell receptor-β^+) $CD4^+$ T cells after 5 days of culture in presence of the indicated treatments. (**b**) Summary of (**a**) normalized to baseline T_{reg} cell conversion. Cross bars indicate the highs and lows of duplicate cultures. All data shown in this figure are representative of at least three independent experiments with similar results.

with control mice (**Figure 6b**). Notably, the reduced inflammatory response was also associated with reduced intestinal and liver pathology (**Figure 6c,d**). These effects were specific to the presence of suppressive motifs as control ns-ODN failed to downregulate inflammatory responses and pathology (see **Supplementary Figure S3** online and data not shown). Further, the sup-ODN used in this experiment specifically limited inflammatory responses mediated by TLR9 signaling as treatment with sup-ODN1 in *Tlr9* $^{-/-}$ mice had no effect (see **Supplementary Figure S4a,b** online). These findings show that treatment of mice with suppressive DNA motifs is sufficient to prevent the development of the catastrophic intestinal immunopathology observed during oral *T. gondii* infection.

To evaluate the suppressive role of sup-ODN in another model of mucosal inflammation, we used the well-established model of DSS-induced colitis. This model of colitis is associated with disruption of epithelial integrity that rapidly leads to acute gut inflammation and weight loss.[36] Strikingly, treatment with sup-ODN1, but not with ns-ODN, significantly limited weight loss associated with DSS administration (**Figure 6e**). Further, colon size was significantly maintained following sup-ODN treatment compared with control-treated animals (**Figure 6f**).

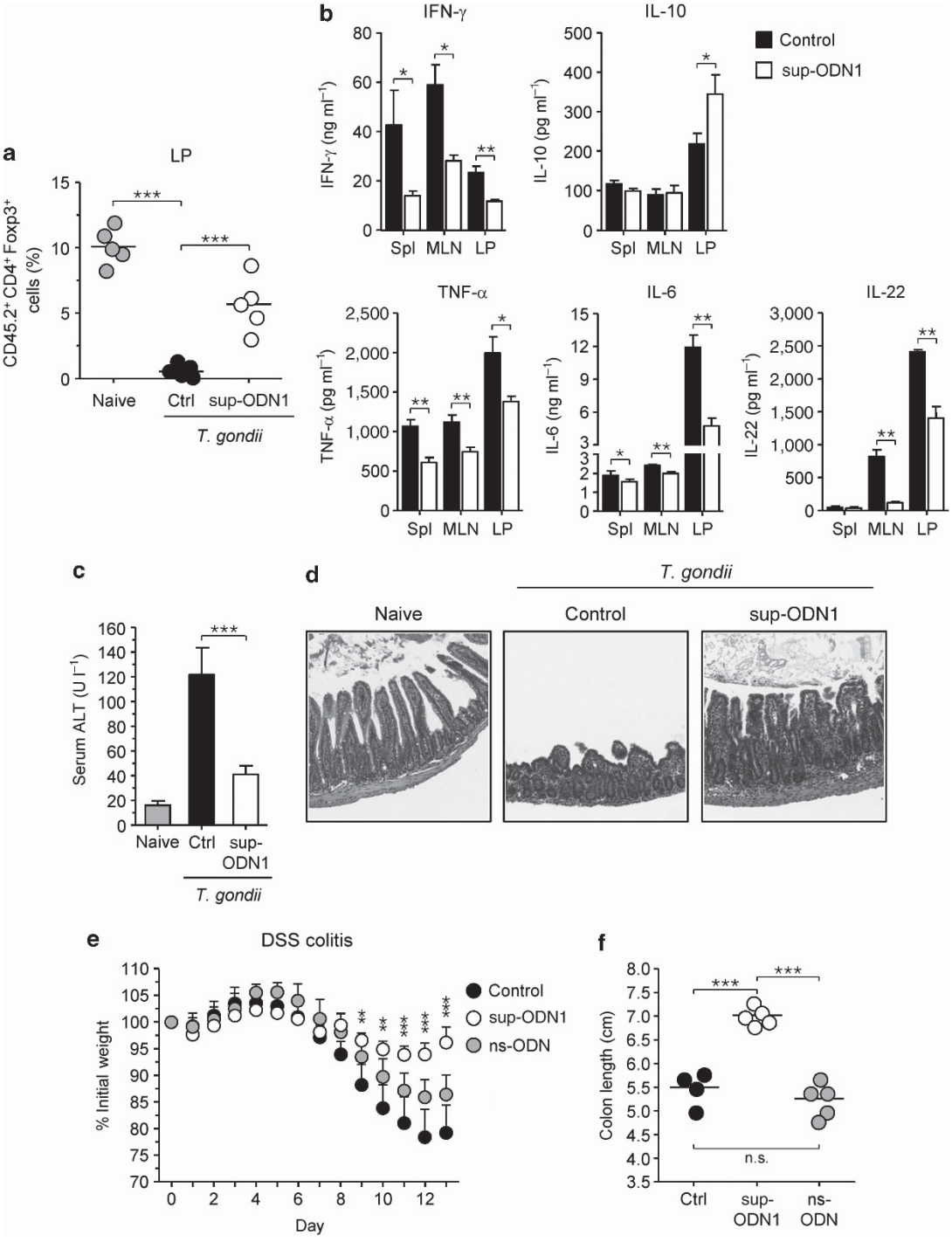
Altogether, these results demonstrate that sup-ODN can limit severe immunopathology in the GI tract.

DISCUSSION

Here we showed that the DNA of defined commensal bacteria can be enriched in suppressive motifs able to exert strong immunoregulatory effects on the mucosal immune system. These findings reveal suppressive DNA motifs as an additional microbiota-derived microbe-associated molecular pattern and support the idea that a balance between stimulatory and regulatory motifs at the commensal DNA level is likely to contribute to the maintenance of gut immune homeostasis and function. We propose a model in which the adjuvant properties of commensal DNA are tightly regulated by suppressive motifs allowing the induction of effector responses without triggering pathogenic responses.

The interaction between the host and the flora relies on a complex network of signals involving numerous bacterial species and microbial-derived ligands. This multitude of signals converges to establish a tonic, regulated but responsive mucosal environment. Indeed, maintenance of responsiveness against pathogenic microbes as well as protection against commensal translocation relies on the establishment of a dynamic equilibrium sustained, in part, by the stimulatory capacity of the flora.[37] Furthermore, recent work demonstrated that defined bacteria (e.g., segmented filamentous bacteria) or bacteria-derived products (e.g., bacterial polysaccharide A, DNA, ATP) could have a dominant role in promoting mucosal immune responses.[6,38–40]

In some instances, defined bacteria species have been associated with enhanced pathogenesis during mucosal inflammation and are over-represented in the gut of patients suffering from inflammatory bowel disease.[41] On the other hand, the microbiota can exert some strong immunoregulatory function via the induction and/or activation of T_{reg} cells at mucosal sites. For instance, *Bacteroides fragilis*–derived polysaccharide A and indigenous bacteria from the genus *Clostridium* can expand or induce T_{reg} cells.[42–44] Recent data also demonstrate that a fraction of mucosal resident T_{reg} are specific for commensal-derived antigens.[45] Further, some bacteria referred to as probiotics have been defined based on their beneficial effect to their host.[8] Promising results have been obtained with probiotics in the treatment of human inflammatory diseases of the intestine and in the prevention and treatment of atopic eczema in neonates and infants.[46,47] The mechanisms behind these functional disparities between commensal species remain to date poorly understood, but has been associated, at least in part, with the induction of various T_{reg}-cell populations. Previous work has identified an association between Crohn's disease and a promotor polymorphism in



the *Tlr9* gene in humans.[48] Such association supports the idea of a role for gut flora DNA sensing in the pathophysiology of inflammatory bowel diseases. We previously showed that the DNA of commensal bacteria can have a profound immunostimulatory role on mucosal immune responses.[6] Strikingly, when used in isolation the DNA of *L. paracasei* is significantly less efficient at inducing immune responses and activating DCs than the DNA of *E. coli*, a commensal known for its stimulatory capacity. However, we found that these differences in the immunostimulatory capacity of *L. paracasei* and *E. coli* DNA were not associated with differences in CpG motifs.

Krieg *et al.*[12] were the first to document the existence of suppressive or neutralizing DNA motifs in the genome of certain strains of adenovirus. Other inhibitory sequences were later characterized in mammalian DNA. In humans and mice, telomeric regions of chromosomes contain large numbers of single-stranded guanosine-rich hexanucleotide repeats of the motif TTAGGG. Synthetic ODNs composed of TTAGGG multimers have been developed and shown to have multiple effects on immune activation.[13,14,23] In particular, these sequences downregulate Th1 responses in the context of CpG ODN stimulation.[11,12,24,25] Importantly, we find that the genomes of bacteria from the *Lactobacillus* genus, including the genome of various probiotic such as *L. paracasei*, are enriched in several suppressive motifs. We find that enrichment in suppressive motifs is a general feature of bacteria from the *Lactobacillus* genus, including bacteria with previously described probiotic activity. Although we cannot state that the relative enrichment of these suppressive motifs represents a unique feature to bacteria with known probiotic properties, we would like to mention that the regulatory functions of all members of the *Lactobacillus* genus have not been thoroughly studied at this time. These results support the idea that enrichment in suppressive DNA motifs is family specific. Our work reveals that these suppressive motifs have a potent immunoregulatory effect on the mucosal immune system. Indeed, these motifs potently inhibit CpG ODN–mediated LpDC activation and sustain mucosal T_{reg}-cell conversion during inflammation both *in vitro* and *in vivo*. We also find that these motifs can limit immunopathology during acute intestinal infection. Complementing our results in an infectious model, we find that suppressive motifs potently inhibit the pathological consequences of DSS-induced colitis as well.

Despite their potent effect on immune responses, the exact mechanism of action of suppressive motifs remains poorly understood. Synthetic oligodeoxynucleotides composed of TTAGGG multimers can block the colocalization of CpG ODN with TLR9 in endosomal vesicles but not the binding or uptake of CpG ODN by immune cells.[11] Further, their immunosuppressive ability depends on their ability to make complex structures and to prevent STAT1, STAT3, and STAT4 phosphorylation.[25,49] Consequently sup-ODN impair the production of various inflammatory mediators such as IL-12 production.[25] Interestingly, other nonimmunogenic nucleotides have been recently shown to strongly suppress CpG-induced innate responses by competing for high mobility group box proteins, known to be essential for triggering all nucleic acid receptor-mediated innate immune responses.[50] Understanding how these motifs inhibit DC activation and immunopathology in the context of TLR9 stimulation in the GI tract remains to be addressed.

Inflammatory bowel disease constitutes a significant health burden in developed countries impacting the quality of life of some 1.4 million individuals in North America and 2.2 million individuals in Europe.[51] There is now clear evidence that the microbiota is a major contributor to the initiation and amplification of mucosal inflammation.[41] Here, we propose that the potential pathogenic outcome associated with microbial dysbiosis may be, in part, associated with increased ratio of immunostimulatory vs. suppressive motifs at the DNA level, an event that could, in genetically predisposed individuals, tip the balance toward inflammation.

Figure 6 Treatment with suppressive oligonucleotides (sup-ODN) restores T_{reg}-cell conversion and decreases intestinal pathology during oral infection with *Toxoplasma gondii* and dextran sulfate sodium (DSS)-induced colitis. (**a**–**d**) C57BL/6 or congenic mice were orally infected or not with *T. gondii*. In addition, some infected mice were orally treated with phosphate-buffered saline (PBS) containing sup-ODN1 (25 μg) every other day, starting 3 days before the infection. Control mice (Ctrl) received PBS only. (**a**) Treatment with sup-ODN1 sustains T_{reg}-cell conversion in mice orally infected with *T. gondii*. At 4 days post-infection, CD45.1$^+$ congenic recipient mice received CD45.2$^+$ CD4$^+$ Foxp3$^-$ OT-II T cells and were then fed with ovalbumin antigen in drinking water for 5 days. On day 9 post-infection, both naive and infected mice were euthanized and TCR-β$^+$ (T-cell receptor-β$^+$) CD4$^+$ CD45.2$^+$-gated T cells from the small intestine *lamina propria* (LP) were assessed for intracellular Foxp3 expression. Each dot represents one mouse and each bar represents the mean of four mice analyzed (**P<0.01; ***P<0.001). (**b**–**d**) Inflammatory immune responses and pathology during *T. gondii* infection are decreased after treatment with sup-ODN1. At day 9 post-infection, mice were euthanized and immune responses, parasite loads, and pathology were assessed. (**b**) Enzyme-linked immunosorbent assay of interferon (IFN)-γ, interleukin (IL)-10, tumor necrosis factor (TNF)-α, IL-6, and IL-22 in supernatants of spleen (Spl), mesenteric lymph nodes (MLN), or small intestine (LP) CD90$^+$ cells prepared from the different groups of mice and restimulated for 48 h with bone marrow-derived dendritic cells loaded with soluble *T. gondii* antigen. Histograms represent the mean cytokine concentration of triplicate wells ± s.d. (*P<0.05; **P<0.01). (**c**) The level of hepatic alanine aminotransferase (ALT) was assessed in the serum of naive or day 9 post-infection mice treated or not with sup-ODN1. Histograms show the mean enzyme concentration ± s.d. (4 mice per group, *P<0.05). (**d**) Histological assessment of ileum from naive and control or sup-ODN1–treated infected mice (hematoxylin and eosin stain, magnification ×100). All experiments shown in this figure are representative of three independent experiments with similar results. (**e, f**) Treatment with sup-ODN1 reduces weight loss and development of colitis upon DSS administration. Mice were given 2% DSS in drinking water for 8 days and changed to normal drinking water on day 9. Some mice received orally PBS containing sup-ODN1 (25 μg) or control ODN (ns-ODN, 25 μg) every other day, from day 0. Control (Ctrl) received PBS only. (**e**) Weight loss measured as percentage of initial weight. Each dot represents the mean weight ± s.d. (4 or 5 mice per group). **P<0.01; ***P<0.001 (sup-ODN1–treated mice compared with control). (**f**) Colon lengths from rectum to cecum were determined at day 13 following DSS treatment. n.s., not significant.

Our findings add an additional layer in our understanding of the complex host–microbiota interaction and propose a model in which the proinflammatory capacity of bacterial DNA can be harnessed by its endogenous content in suppressive motifs. Further, our data support the idea that sup-ODN may represent an important therapeutic approach in the treatment of mucosal inflammatory responses.

METHODS

Mice. C57BL/6 (WT) and B6 SJL mice were purchased from Taconic Farms. B6.129P2-*Tlr9*^{tmAki} (*Tlr9* $^{-/-}$)[52] mice were obtained from Dr S. Akira (Osaka University, Japan) via Dr R. Seder (Vaccine Research Center, NIH) and backcrossed 11 generations onto the C57BL/6 background (Taconic, Germantown, NY). Foxp3 eGFP reporter mice (Foxp3^{eGFP}) were obtained from Dr M. Oukka (Seattle Children's Research Institute).[26] *Tlr9* $^{-/-}$ Foxp3^{eGFP} mice were generated by crossing the F1 progeny of *Tlr9* $^{-/-}$×Foxp3^{eGFP} breeders. We generated OT-II Tg×Foxp3^{eGFP} mice. All mice were bred and maintained under pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the NIAID and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals. All experiments were performed at the NIAID under an animal study proposal approved by the NIAID Animal Care and Use Committee. Mice between 6 and 12 weeks of age were used. Mice were sex- and age-matched for each experiment.

Parasite and infection protocol. A rabbit isolate of *E. cuniculi* obtained from Waterborne (New Orleans, LA) was used throughout the study. The parasites were maintained by continuous passage in rabbit kidney RK13 cells (ATCC CCL-37). Spores were collected from the culture medium, resuspended in sterile phosphate-buffered saline (PBS) pH 7.2, and immediately used for inoculation of mice or cell cultures. Mice were infected by intragastric gavage with 5×10^6 fresh spores.

ME-49 clone C1 of *T. gondii* (kindly provided by Dr ME Grigg, NIAID/NIH) was obtained by electroporation of the parental ME-49 type II strain (ATCC 50840) with the red fluorescent protein and was used for production of tissue cysts in C57BL/6 mice. Tissue cysts used in experiments were obtained from female mice that were perorally inoculated with 10 cysts 2 months earlier. Animals were killed and their brains removed and homogenized in 1 ml of PBS pH 7.2. Cysts were counted under an inverted fluorescent microscope on the basis of two aliquots of 20 µl. For experiments, female mice were infected by intragastric gavage with 10 cysts of ME-49 C1.

Quantitation of parasite tissue loads. For experiments with *E. cuniculi*, duodenum was removed from infected mice and digested with proteinase K (Qiagen, Los Angeles, CA) after homogenization. DNA was subsequently extracted with the DNeasy Tissue kit from Qiagen. Quantitative real-time PCR was performed in triplicate with 50 ng of total tissue DNA, the iQ SYBR Green Supermix (BioRad, Richmond, CA), and the following primers specific for a 268-bp DNA sequence of the SSU rRNA gene from *E. cuniculi*: forward 5′-GTGAGACCCTTTGACGGTGT-3′ and reverse 5′-CTCAGACCTTCCGATCTTCG-3′. Real-time PCR was conducted on a Bio-Rad iCycler under the following conditions: 3 min at 95 °C; 40 cycles of 45 s at 95 °C; 60 s at 60 °C; and 45 s at 72 °C. Genomic DNA was extracted from known amounts of *E. cuniculi* spores with the QIAamp DNA stool mini-kit (Qiagen) and used as PCR standards. A standard curve was generated by linear regression on plotted cycle threshold (C_T) values of the standards against the logarithms of parasite numbers using iCycler iQ Optical System software (version 3.1; Bio-Rad).

Hs27 human fibroblast (ATCC CRL-1634) cultures were used for parasite burden quantitation following infection with *T. gondii*. Tissue single-cell suspensions (10^4–10^6 cells) were added onto confluent fibroblast monolayers and titrated by looking at plaque formation under an inverted fluorescent microscope 6 to 8 days later. Results of these titrations are reported as plaque-forming units.

Genomic DNA extraction from bacteria. *E. coli* strain K12 substrain MG1655 was grown in tryptic soy broth medium (Fluka, St Louis, MO) under aerobic conditions at 37 °C with constant shaking. *L. paracasei* strain ATCC 25302 was grown in Lactobacilli MRS broth (Hardy Diagnostic, Santa Maria, CA) under anaerobic conditions at 37 °C. Overnight cultures of *E. coli* or *L. paracasei* were centrifuged at 3,300 *g* and 4 °C for 20 min. Pellets were resuspended in lysis buffer (10 mM Tris/HCl, 50 mM ethylenediaminetetraacetic acid, pH 8.0) containing lysozyme (0.5 mg ml^{-1}; Sigma-Aldrich, St Louis, MO). After incubation at 37 °C for 2 h, 2 mg ml^{-1} of proteinase K and 1% sodium dodecyl sulfate were added and the sample incubated at 60 °C for 3 h. DNA was then purified by a series of seven consecutive phenol–chloroform–isoamyl alcohol affinity extractions. The quality and the amount of purified DNA were measured and quantified using a NanoDrop 3300 spectrophotometer (Thermo Scientific, Wilmington, DE).

ODN. ODN with a nuclease-resistant phosphorothioate backbone were custom synthesized at the Center for Biologics Evaluation and Research core facility (Food and Drug Administration, Bethesda, MD). The following ODNs were used in experiments: stimulatory CpG ODN 1555, GCTAGACGTTAGCGT; sup-ODN A151 (sup-ODN1) TTAGGGTTAGGGTTAGGGTTAGGG;[23] sup-ODN H154 (sup-ODN2) CCTCAAGCTTGAGGGG;[24] and ns-ODN, GTCACGGCTGATGAGCG. ODNs contained < 0.1 units of endotoxin per mg, as assessed by a Limulus amebocyte cell lysate assay. CpG ODN 1826 was purchased from Coley Pharmaceutical (Wellesley, MA). For injection in mice, all ODNs were diluted in PBS pH 7.2 and mice were orally injected with isotonic bicarbonate buffer 10 min before gavage with ODNs.

Bioinformatics. Genome data were downloaded from NCBI Genomes database for 28 different species of *Lactobacillus* bacteria and 27 strains of *E. coli* (see **Supplementary Tables 1** and **2** online). The presence of the following sequence motifs in the genome of different bacteria was analyzed: AACGTT and ATCGAT for the CpG motifs and TTAGGG and TCAAGCTTGA for the suppressive motifs.[23,24] "Perl" scripts were used to match the motif patterns to the bacteria sequences. Alternatively, the motifs were enumerated using the "fuzznuc" program of the EMBOSS package (http://emboss.sourceforge.net). Custom PHP scripts were written to extract and tabulate the results obtained using the above programs.

ATB treatment. Male, 3-week-old C57BL/6 mice were provided ampicillin (1 g l^{-1}), neomycin trisulfate (1 g l^{-1}), metronidazole (1 g l^{-1}), and vancomycin (500 mg l^{-1}) in drinking water as previously described.[3] All ATBs were purchased from Sigma-Aldrich (St Louis, MO). Six weeks after the start of the ATB course, mice also received orally various treatments once a week: CpG ODN 1826 (100 µg) in sterile PBS, DNA from *E. coli* (500 µg), or DNA from *L. paracasei* (500 µg) in sterile water. Two weeks later, mice were infected with *E. cuniculi* by gavage. In some experiments, mice were treated orally every 3 days with CpG ODN 1826 (25 µg) alone or in combination with sup-ODN1 (25 µg) starting 6 weeks after the beginning of the ATB regimen. Mice were then gavaged with *E. cuniculi* 6 days later.

***In vitro* restimulation and intracellular cytokine detection**. RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 100 µg ml^{-1} gentamicin, 50 mM of β-mercaptoethanol, 1 mM sodium pyruvate and nonessential amino acids (complete medium) was used for *in vitro* restimulation. Single-cell suspensions of leukocytes from spleen, mesenteric lymph nodes and small intestinal LP of naive or infected mice were prepared as previously reported.[22] For experiment with *E. cuniculi*, 5×10^5 leukocytes were co-cultured with 1×10^5 bone marrow-derived DC in a 96-well U-bottom plate. Bone marrow-derived DC were previously incubated overnight with or without *E. cuniculi* spores (parasite:bone marrow-derived

DC ratio, 10:1) in the presence of 20 ng ml^{-1} granulocyte macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ) and washed before culture with leukocytes. For experiment with *T. gondii*, leukocytes were stained with the CD90.2-positive selection kit and enriched for T cells with an autoMACS Pro (Miltenyi Biotec, Auburn, CA). After enrichment, 2.5×10^5 T cells were then co-cultured with 5×10^4 irradiated (3,000 rad) bone marrow-derived DC and with or without soluble *T. gondii* antigen (STAg, 5 μg ml^{-1}) in a 96-well U-bottom plate. After 2 or 3 days at 37 °C in 5% CO_2, culture supernatants were collected for cytokine assays. IFN-γ, IL-6, IL-10, IL-17, IL-22, and/or TNF-α were quantitated in culture supernatants using the DuoSet ELISA system (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. For intracellular cytokine detection, LP single-cell suspensions were cultured in triplicate at 2×10^6 cells per milliliter in a 96-well U-bottom plate and stimulated with various amounts of CpG ODN 1826, CpG ODN 1555, DNA from *E. coli* or *L. paracasei*, sup-ODN1, and/or ns-ODN for 5 h at 37 °C in 5% CO_2. Brefeldin A (GolgiPlug, BD Biosciences, San Jose, CA) was added for the final 4 h of culture. After restimulation, cells were incubated with purified anti-mouse CD16/32 (clone 93) and with fluorochrome-conjugated antibodies against surface markers CD11b (M1/70), CD11c (N418), MHCII (M5/114.15.2), F4/80 (BM8), and CD103 (2E7), in Hank's buffered salt solution for 20 min at 4 °C and then washed. LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) was used to exclude dead cells. Cells were then fixed for 15 min at room temperature using 2% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA), washed twice, and then stained with fluorochrome-conjugated antibodies against IL-12/23p40 (C15.6), TNF-α (MP6-XT22), or isotype control rat IgG1κ (R3-34) for 30 min at 4 °C in permeabilization buffer supplied with the BD Cytofix/Cytoperm kit (BD Biosciences) containing anti-mouse CD16/32, 0.2 mg ml^{-1} purified rat IgG and 1 mg ml^{-1} of normal mouse serum (Jackson Immunoresearch, West Grove, PA). All antibodies were purchased from eBioscience (San Diego, CA) or BD Biosciences. Cell acquisition was performed on an LSRII flow cytometer using FACSDiVa software (BD Biosciences). For each sample, at least 300,000 events were collected. Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

LpDC and T cell purification. LpDCs were purified from the small intestine of naive wild-type or *Tlr9* $^{-/-}$ mice. After LP digests were passed through 70- and 40-μm cell strainers, cells were resuspended in 1.077 g cm^{-3} iso-osmotic animal cell separation medium (Accurate Chemical & Scientific, Westbury, NY), and overlayed with RPMI 1640 medium. After centrifugation at 1,650 *g* for 15 min, cells were collected from the low-density fraction, washed, and then incubated with a mixture of antibodies containing α-CD16/32 (2.4G2), α-CD11c (HL-3), and α-MHCII (AF6-120.1), as well as the non-DC components α-TCR-β (H57-597) and 7-AAD viability staining solution (all from eBioscience). DCs were defined as TCR-β$^-$ CD11c^{hi} MHCII$^+$ cells and sorted by flow cytometry on a FACSAria (BD Biosciences). The post-sort purity was higher than 95%. Purified CD11c^{hi} MHCII$^+$ cells were used for *in vitro* conversion assay or stimulated for 18 h with CpG ODN 1826, DNA from *E. coli* or *L. paracasei*, sup-ODN1, and/or non-sense ODN in the presence of granulocyte macrophage colony-stimulating factor (50 ng ml^{-1}, Peprotech) in 5% CO_2. For T-cell purification, single-cell suspensions of spleen and peripheral lymph nodes extracted from *Tlr9* $^{-/-}$ Foxp3^{eGFP} mice were enriched for CD4$^+$ T cells by negative selection using an autoMACS Pro (Miltenyi Biotec). The enriched fraction was further labeled with fluorochrome-conjugated antibodies against CD4 (RM4-5), CD25 (7D4), and CD44 (IM7); all from eBioscience), and sorted by flow cytometry on a FACSAria. Purified CD4$^+$ CD25$^-$ CD44^{lo} Foxp3$^-$ T cells (<0.5% Foxp3$^-$) were used for *in vitro* conversion assays.

***In vitro* conversion assay**. Our conversion protocol was performed as previously reported[26] with T cells and LpDCs obtained as described above. In brief, FACS (fluorescence-activated cell sorter)-purified LpDCs and CD4$^+$ T cells were co-cultured at a 1:10 ratio (1×10^5 CD4$^+$ T cells) in complete medium and Treg cell–polarizing conditions: soluble α-CD3 (1 μg ml^{-1}, BD Biosciences) and human recombinant TGF-β (0.3 ng ml^{-1}, R&D Systems). Co-cultures were supplemented with 5 ng ml^{-1} of recombinant human IL-2 (Peprotech) every 2 days. In some experiments, CpG ODN 1555, sup-ODN1, ns-ODN, *E. coli* DNA, and *L. paracasei* DNA were added individually or in combination at various concentrations at the start of the co-cultures in Treg cell–polarizing conditions. On day 5, cells were stained with the LIVE/DEAD Fixable Blue Dead Cell Stain Kit and fluorochrome-conjugated antibodies against the cell-surface markers CD4 (RM4-5) and $\alpha_4\beta_7$ (DATK32; all from eBioscience) in Hank's buffered salt solution for 20 min at 4 °C and then washed twice. Foxp3 staining was subsequently performed using the Foxp3 staining set (eBioscience) according to the manufacturer's protocol.

Oral antigen administration. B6.SJL (CD45.1) mice were orally infected or not with 10 ME-49 C1 cysts of *T. gondii*. CD4$^+$ Foxp3$^-$ T lymphocytes from the secondary lymph nodes and spleen of naive OT-II Tg×Foxp3^{eGFP} (CD45.2) mice were purified by cell sorting and adoptively transferred into naive or infected (4 days post infection) B6.SJL recipient mice. Each mouse received 1.5×10^6 cells and was then fed with ovalbumin dissolved in drinking water (1.5% solution; grade V from Sigma-Aldrich) for 5 consecutive days. On day 9 after infection, mesenteric lymph nodes and LP single-cell suspensions were prepared from B6.SJL hosts as described above and intracellular Foxp3 expression was assessed among CD45.1$^+$ transferred cells. In some experiments, B6.SJL recipient mice were injected by gavage with 25 μg of sup-ODN1 on days −3 and −1 before infection with *T. gondii* and on days 1, 3, 5, and 7 post-infection.

Pathology assessment. Mice treated or not with sup-ODN1 were euthanized 9 days post-oral infection with *T. gondii*. The ileum from each mouse was removed and immediately fixed in PBS containing 10% formalin. Paraffin-embedded sections were cut at 0.5 mm, stained with hematoxylin and eosin and examined histologically. Liver alanine aminotransferase levels were measured in serum samples, using commercially available kits (Boerhinger Mannheim, Indianapolis, IN).

Induction of DSS colitis. Mice were given 2% (w/v) DSS (M.W. = 36,000–50,000 kDa, MP Biologicals, Solon, OH) in their drinking water for 8 days, and subsequently switched to regular drinking water. The amount of DSS water drank was recorded for each cage and no differences in intake between cages were observed. Mice were weighed daily. Some mice were also treated orally every other day with sup-ODN1 (25 μg) or ns-ODN (25 μg) starting at day 0 and for the duration of the experiments. Mice were euthanized on day 13 and colons were removed, flushed, and the length was measured from rectum to cecum.

Statistical analysis. Groups were compared with Prism software (GraphPad) using the two-tailed unpaired Student's *t*-test. Data are presented as mean ± s.d. $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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