Intestinal inflammation alters mucosal carbohydrate foraging and monosaccharide incorporation into microbial glycans

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Abstract
Endogenous carbohydrates released from the intestinal mucus represent a constant source of nutrients to the intestinal microbiota. Mucus-derived carbohydrates can also be used as building blocks in the biosynthesis of bacterial cell wall components, thereby influencing host mucosal immunity. To assess the uptake of endogenous carbohydrates by gut microbes in healthy mice and during intestinal inflammation, we applied azido-monosaccharides that can be tracked on bacterial cell walls after conjugation with fluorophores. In interleukin-10 deficient mice, changes in the gut microbiota were accompanied by decreased carbohydrate hydrolase activities and increased lumenal concentrations of host glycan-derived monosaccharides. Tracking of the monosaccharide N-azidoacetylglucosamine (GlcNAz) in caecum bacteria revealed a preferential incorporation of this carbohydrate by Xanthomonadaceae in healthy mice and by Bacteroidaceae in interleukin-10 deficient mice. These GlcNAz-positive Bacteroidaceae fractions mainly belonged to the species B. acidifaciens and B. vulgatus. Growth of Bacteroides species in the presence of specific monosaccharides changed their stimulatory activity toward CD11c+ dendritic cells. Expression of activation markers and cytokine production was highest after stimulation of dendritic cells with B. vulgatus. The variable incorporation of monosaccharides by related Bacteroides species underline the necessity to investigate intestinal bacteria down to the species level when addressing microbiota-host interactions.

KEYWORDS
bacteria, Bacteroides, carbohydrate hydrolase, click chemistry, dendritic cell, GlcNAc, Glycan, interleukin-10, mucin, mucus

1 | INTRODUCTION

The gut microbiota is an inherent component of animal physiology that influences the functions of multiple organ systems. The composition of the gut microbiota is itself under the influence of exogenous factors such as dietary intake, ingested drugs, and endogenous factors such as the immune system and the intestinal mucosa (Weiss & Hennet, 2017). The mucus lining of the gastrointestinal tract provides a protective barrier preventing the direct contact of the epithelium with microbes. This mucus, which is mainly constituted of heavily
glycosylated mucin proteins (Davies et al., 2016), also represents a source of carbohydrates used by several intestinal microbes as nutrients.

The secretion of mucins and the resulting thickness of the mucus layer varies along the segments of the gastrointestinal tract. The mucus is thin and patchy in the small intestine, while thick and stratified in the large intestine that harbours most of the microbiota. The mucus plays an ambivalent role in the relationship of the host to its microbiota. Whereas the stratified inner mucus layer acts as a barrier (Johansson et al., 2008), the outer gel-like layer creates a bacterial habitat, which enables the microbial foraging of host glycans (Hansson, 2012). Bacterial lipopolysaccharide (LPS), a relative expansion of Proteobacteria (Lupp et al., 2007; Maharshak et al., 2013). The resulting changes in microbial composition are likely to affect bacterial metabolic and biosynthetic pathways, such as the extraction and utilisation of mucosal carbohydrates by specific bacterial taxa. Already at 8–10 weeks of age, Il10−/− mice presented a significant colon inflammation featuring shortening of colon length, leukocyte infiltration, local pro-inflammatory cytokine secretion, cryptitis, loss of goblet cells and epithelial damage (Grabinger et al., 2019). At this stage, we assessed changes in mucosal carbohydrate extraction by measuring the major carbohydrate hydrolase activities in the caecum of healthy WT mice and colitic Il10−/− mice using colorimetric assays. Some activities, such as α-galactosidase, α- and β-N-acetylgalactosaminidase, α- and β-fucosidase were decreased in Il10−/− mice, while β-galactosidase and α-sialidase activities remained unchanged (Figure 1a). Given the decrease in carbohydrate hydrolases found in Il10−/− mice, we expected a similar decrease in the luminal levels of monosaccharides released into the caecum. Surprisingly, we detected increased concentrations of the monosaccharides Gal, GalNAc, GlcNAc, Fuc, and the sialic acids N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2), but not glucose (Glc) in Il10−/− mice (Figure 1b). Whereas Glc is mainly derived from dietary fibres, the other monosaccharides represented common building blocks of mucin O-glycans (Koropatkin, Cameron, & Martens, 2012; Thomson et al., 2012). The monosaccharide galactosamine (GalN) was also increased in the caecum lumen of Il10−/− mice, although this carbohydrate is not a constituent of mucin O-glycans.

The concomitant decreased carbohydrate hydrolase activity and increased monosaccharide concentrations pointed to a shift in carbohydrate foraging in Il10−/− mice. The increased availability of monosaccharides from intestinal glycans likely influenced the composition of the gut microbiota by providing additional nutrients to specific bacterial taxa. To identify the bacterial groups mainly affected by the increased monosaccharide concentrations, we focused on GlcNAc, which showed the highest increase among the mucin-derived carbohydrates in the caecum lumen (Figure 1b).

2 | RESULTS

2.1 | Mucosal glycan utilisation in caecum of WT and Il10−/− mice

IL-10 suppresses the biosynthesis of pro-inflammatory cytokines by macrophages, natural killer cells and T cells. Mice lacking Il10 develop enterocolitis due to the failure to dampen the continuous stimulation of the intestinal immune system by enteric antigens (Kuhn, Lohler, Rennick, Rajewsky, & Muller, 1993). Colonic inflammation in Il10−/− mice is accompanied by a loss of microbial diversity and richness paralleled with a relative expansion of Proteobacteria (Lupp et al., 2007; Maharshak et al., 2013). The resulting changes in microbial composition are likely to affect bacterial metabolic and biosynthetic pathways, such as the extraction and utilisation of mucosal carbohydrates by specific bacterial taxa.

In addition to their nutritional value, mucin-derived carbohydrates can also be used as building blocks in the biosynthesis of bacterial cell wall components such as peptidoglycan, lipo- and capsular polysaccharides, which contribute to the activation of host immunity. Alternatively, carbohydrates can also be incorporated into cell wall structures to evade immune recognition through molecular mimicry. For example, Neisseria spp. and Haemophilus influenzae can integrate sialic acid into their lipooligosaccharides to imitate human antigens (Harvey, Swords, & Apicella, 2001) and Staphylococcus aureus employs protein modifications with GlcNAc to escape host defences (Thomer et al., 2014).

Despite the established effects of mucosal carbohydrates on the intestinal microbiota (Huang, Chassard, Hausmann, von Itzstein, & Hennet, 2015; Ng et al., 2013), little is known about the identity of bacterial taxa incorporating mucin-derived carbohydrates into their cell wall. To address this question, we applied traceable azido carbohydrates in the caecum lumen (Figure 1b).

2.2 | Incorporation of exogenous monosaccharides by microbial communities

To assess the impact of GlcNAc on the intestinal microbiota, the natural monosaccharide was used as well as its azido derivative to enable
FIGURE 1  Carbohydrates released in caecum fluid of WT and Il10−/− mice. (a) Carbohydrate hydrolase activities in 8–10 weeks old mice were measured in caecum fluid by colorimetric assays. Data are shown as mean and standard deviation from 4 to 5 mice per group. 

(b) Monosaccharide concentrations of core and terminal glycan monosaccharides were determined by HPLC. Glucosamine was not detected in any of the samples. Fuc, fucose; Gal, galactose; GalN, galactosamine; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Neu5Ac, 5-N-acetylneuraminic acid; Neu5,9Ac2, 5-N-acetyl-9-O-acetyleneuraminic acid; Neu5Gc, 5-N-glycolylneuraminic acid. Data represent the mean and standard deviation from 5 to 6 mice investigated at different time points. Statistical significance was determined by unpaired t-test (*, p < .05)
the subsequent identification of bacteria having incorporated this monosaccharide on their surface. After isolation of the caecum microbiota from mice under oxygen free conditions (Hungate, 1969), we compared the effect of GlcNAc and its azido derivative N-azidoacetylglucosamine (GlcNAz) on the microbial composition by supplementing anaerobic bacterial cultures with GlcNAc, GlcNAz or Glc as control. The bacterial composition was analysed by 16S rRNA sequencing before and after 6 hr of culture, which resulted in an increase of OD600 by 0.5–0.6. This short incubation time was chosen to minimise the impact of different bacterial growth rates over the incorporation of exogenous monosaccharides. As expected, the composition of the gut microbiota differed between WT and Il10−/− mice, with increased relative abundance of Bacteroidaceae and Ruminococcaceae, and decreased relative abundance of Helicobacteraceae in Il10−/− mice (Figure 2). Compared with Glc, supplementation of the caecum microbiota with GlcNAc and GlcNAz did not change the microbiota composition significantly in both genotypes when assessed at the family level (Figure 2).

The application of GlcNAz enabled its subsequent labelling after ligation with alkyne-containing fluorophore using click chemistry (Agard, Baskin, Prescher, Lo, & Bertozzi, 2006). Incubation of caecum microbiota with GlcNAz resulted in the incorporation of this monosaccharide on the surface of a portion of the bacterial community as detected by flow cytometry after ligation of the fluorophore AlexaFluor488-alkyne to the azido group of GlcNAz (Figure 3). Because GlcNAc, and concomitantly GlcNAz, can be converted to other monosaccharides after uptake by bacteria, we also investigated the incorporation of N-azidoacetylmannosamine (ManNAz) and N-azidoacetylsialic acid (SiaNAz), which are two possible products of GlcNAz interconversion. These two monosaccharides were also incorporated into caecum bacteria from WT and Il10−/− mice as evidenced by increased AlexaFluor488 labelling and flow cytometry (Figure 3). The incorporation of ManNAz was strongest in WT caecum bacteria, whereas SiaNAz was nearly equally incorporated in bacteria from both genotypes.

To identify the caecum bacteria that incorporated GlcNAz, we sorted AlexaFluor488-labelled bacteria from WT and Il10−/− derived caecum samples incubated with GlcNAz for 6 hr. The fraction of AlexaFluor488-positive bacteria increased by 6.5-fold and by 8.9-fold in WT and Il10−/− samples after incubation with GlcNAz over GlcNAc, respectively. The composition of these AlexaFluor488-positive bacteria was determined by 16S rRNA sequencing. In bacteria derived from WT mice, the families of Xanthomonadaceae and Pseudomonadaceae were significantly enriched among GlcNAz-positive bacteria. By contrast, in Il10−/− mice, the family of Bacteroidaceae was the most abundant in GlcNAz-positive samples, reaching up to 64% of all bacteria (Figure 4a). Strikingly, the distribution of Bacteroidaceae differed in the two genetical backgrounds. In samples from Il10−/− mice, GlcNAz-positive and GlcNAz-negative bacteria did not differ in the number of

**FIGURE 2** GlcNAc and GlcNAz supplementation of the intestinal microbiota induces similar changes as supplementation with Glc. Cultures of caecum slurries from WT and Il10−/− mice were supplemented with 1 mM GlcNAc, 1 mM GlcNAz or 1 mM Glc for 6 hr. Microbial changes were analysed by next generation sequencing of 16S rRNA. Graph depicts sequencing analysis at the family level of caecum content and supplemented cultures. Only bacterial taxa representing >1% of total identified sequences are presented. Data show the average percentage of total identified sequences obtained from three mice per group investigated at distinct time points. Bacteroidaceae, Ruminococcaceae, and Helicobacteraceae were significantly different between WT and Il10−/− mice in the starting caecum contents as determined by analysis of variance with Bonferroni post hoc test, p < .001.
bacterial families, but in the abundance of each family. Principal coordinates analysis confirmed that the differences between the two sorted bacterial groups did not occur in respect to the number of identified OTUs, but due to the unequal abundances of the OTUs in the two groups. This can be seen through the clustering of weighted Unifrac distances along principal coordinate 1 in the \( \text{Il10}^{-/-} \) background. In WT mice, weighted Unifrac distances did not yield GlcNAz-specific clusters (Figure S1). Closer examination of the bacteria identified in the GlcNAz-positive fractions revealed that all members of the Bacteroidaceae family belonged to the genus Bacteroides with \( B. \) acidifaciens and \( B. \) vulgatus being the dominant species (Figure 4b). The distribution of these two Bacteroides species in WT and \( \text{Il10}^{-/-} \) mouse samples was validated by real-time quantitative PCR using primers specific for the recA gene of \( B. \) acidifaciens and the sialidase gene \( bv-J266 \) of \( B. \) vulgatus. The constant levels of \( B. \) vulgatus in WT samples and the increased abundance of \( B. \) vulgatus and \( B. \) acidifaciens in \( \text{Il10}^{-/-} \) samples enriched for GlcNAz incorporation was confirmed by the PCR analysis (Figure S2).

2.3 | Incorporation of monosaccharides by Bacteroides species

Several Bacteroides are efficient carbohydrate foragers (Martens et al., 2011), although the expression of carbohydrate hydrolases and carbohydrate transporters varies between Bacteroides species (Ng et al., 2013). We therefore compared the uptake and incorporation of GlcNAz with pure cultures of \( B. \) acidifaciens and \( B. \) vulgatus. We included two additional Bacteroides species, \( B. \) thetaiotaomicron and \( B. \) intestinalis, and we also compared the incorporation of GlcNAz with that of ManNAz and SiaNAz in the four bacterial species. These azido-monosaccharides were added to growth medium containing Glc as a main carbohydrate source. The incorporation of the monosaccharide derivatives was quantified by flow cytometry after reaction of AlexaFluor488-alkyne with azido groups by click chemistry. GlcNAz was efficiently incorporated into surface glycans of the four Bacteroides species investigated (Figure 5). This finding supports the notion that these Bacteroides species compete for GlcNAz as a carbon source.

**FIGURE 3** Surface presentation of azido sugars of caecum microbiota. Caecum slurries of WT and \( \text{Il10}^{-/-} \) mice were cultured anaerobically and supplemented with GlcNAc, GlcNAz, ManNAz or SiaNAz for 6 hr, then stained with A488-labelled alkyne dye prior to analysis by flow cytometry. Azido sugar-supplemented bacteria not labelled with alkyne dye were used as a second negative control. GlcNAc-supplemented bacteria (red lines), GlcNAz-supplemented bacteria (alkyne-A488 labelled, dark blue; unlabelled, light blue lines), ManNAz-supplemented bacteria (alkyne-A488 labelled, dark green; unlabelled, light green lines), SiaNAz-supplemented bacteria (alkyne-A488 labelled, dark orange; unlabelled, light orange lines). The data shown are representative of three independent experiments.
and building block for surface glycans. The incorporation of ManNAz was more restricted, given that B. acidifaciens hardly presented any ManNAz on its surface while B. thetaiotaomicron and B. vulgatus efficiently presented this monosaccharide derivative on their surface. The incorporation of SiaNAz in surface glycans was even more selective, as only B. vulgatus presented this monosaccharide significantly among the four Bacteroides tested (Figure 5). Growth of B. vulgatus and B. acidifaciens in the absence of Glc in M9 minimal medium demonstrated that both Bacteroides species could grow on GlcNAc, whereas B. vulgatus could also utilise SiaNAc as carbon sources. By contrast, B. acidifaciens could only grow in minimal medium including Glc or GlcNAc. Both species did not proliferate when ManNAz was available as single carbohydrate source (Figure 6). Incorporation of GlcNAz, ManNAz, and SiaNAz was also investigated in other commensal bacteria including Escherichia coli, Lactobacillus brevis, Lactobacillus plantarum, Akkermansia muciniphila and Barnesiella intestinihominis. These bacteria however did not expose any of the three monosaccharide derivatives tested on their surface (data not shown).

2.4 Differential activation of dendritic cells by Bacteroides species

The selective proliferation of individual Bacteroides species in response to monosaccharide availability is likely to influence host immunity, thus possibly affecting the severity of intestinal

| GlcNAz label | WT | Il10−/− |
|--------------|----|---------|
| +            | 0  | 0       |
| -            | 100| 100     |

![FIGURE 4 Composition of azide-label-positive and azide-label-negative bacteria.](image)

(a) 16S rRNA sequencing analysis of GlcNAz-supplemented caecum bacteria sorted as azide-label-positive and azide-label-negative. Sequencing data from WT and Il10−/− mice are shown at family level. Only bacterial taxa representing >1% of total identified sequences are presented. Data show the average percentage of total identified sequences obtained from three mice per group investigated at different time points. Pseudomonadaceae and Xanthomonadaceae were significantly different between GlcNAz-negative and GlcNAz-positive in WT mice and Bacteroidaceae were significantly different between GlcNAz-negative and GlcNAz-positive in Il10−/− mice as determined by analysis of variance with Bonferroni post hoc test, p < .001. (b). Relative abundance of individual Bacteroides species among Bacteroidaceae identified by 16S rRNA sequencing analysis in WT- and Il10−/−-derived GlcNAz-supplemented and sorted (GlcNAz label +/−) caecum bacteria from WT and Il10−/− mice investigated independently (3 mice per genotype). The relative abundance of the Bacteroides species in the original caecum contents expressed against total bacteria is indicated as “caecum content.”
inflammation in Il10−/− mice. To assess the effects of monosaccharide incorporation by Bacteroides on immune cell functions, we stimulated bone marrow-derived CD11c+ dendritic cells with fixed B. vulgatus and B. acidifaciens grown in medium supplemented with GlcNAc, ManNAc and SiaNAc. With increasing GlcNAc and SiaNAc concentrations, B. vulgatus showed a decreased ability to induce the expression of the activation markers CD40 and CD86 in dendritic cells when compared to bacteria grown in glucose (Figure 7a). By contrast, B. acidifaciens hardly induced CD40 and CD86 expression when presented to dendritic cells. Growth in GlcNAc- and SiaNAc containing medium only slightly increased the stimulatory properties of B. acidifaciens in respect to CD40 and CD86 expression, respectively.
The direct stimulation of CD11c+ dendritic cells with purified LPS from *B. vulgatus* and *B. acidifaciens* (Figure S3) cultured in presence of monosaccharides at 20 mM confirmed the differential impact of GlcNAc, ManNAc and SiaNAc supplementation on the stimulatory potential of *B. vulgatus* LPS. As shown for the activation of dendritic cells by whole bacteria, the stimulatory activity of LPS from *B. acidifaciens* was less affected by GlcNAc, ManNAc and SiaNAc supplementation (Figure 7c).
The stimulation of CD11c+ dendritic cells with *B. vulgatus* grown at elevated GlcNAc- and SiaNAc concentrations also decreased the production of the pro-inflammatory cytokines TNFα and IL-1β when compared to dendritic cells stimulated with *B. vulgatus* grown in glucose-containing medium (Figure 8a). Surprisingly, IL-1β secretion was increased by stimulation with *B. vulgatus* grown at high ManNAc concentration (20 mM) compared to *B. vulgatus* grown in 5 mM ManNAc. Growth at 20 mM GlcNAc or ManNAc also lowered the production of IL-10 in dendritic cells. Elevated monosaccharide concentrations had the opposite effect on IL-17A production stimulated by *B. vulgatus*, an effect that was clearest for GlcNAc and SiaNAc (Figure 8a). As observed for the expression of the activation markers CD40 and CD86, *B. acidifaciens* only induced low levels of TNFα, IL-1β and IL-10 production compared to *B. vulgatus* (Figure 8b). Interestingly, *B. acidifaciens* induced the secretion of IL-17A at similar levels as *B. vulgatus*, although the prior growth of *B. acidifaciens* at varying monosaccharide concentrations did not affect the stimulation of IL-17A production significantly (Figure 8b). These findings

**FIGURE 8** Availability of specific carbohydrate substrates in *Bacteroides* cultures leads to a differential cytokine expression profile in bone marrow-derived dendritic cells. Cultures of *B. vulgatus* (a) or *B. acidifaciens* (b) were grown 16 hr in the presence of GlcNAc, ManNAc or SiaNAc in various concentrations (5 and 20 mM) or Glc (20 mM). Harvested bacteria were washed, PFA-fixed and used for a stimulation of dendritic cells for 24 hr with a bacteria-to-cell ratio of 10:1. Unstimulated (PBS) and *E. coli* O111:B4 LPS-stimulated dendritic cells served as controls. Expression of the cytokines TNFα, IL-1β, IL-10 and IL-17A was determined in supernatants by a multiplex immunoassay. Cytokine concentration in pg/ml is presented as mean values and standard deviation (n = 3) from independent experiments. Statistical significance (p < .05) as indicated by an asterisk was determined by one-way analysis of variance with a Bonferroni post-hoc test.
therefore underlined the impact of mucosal monosaccharide availability on the selective expansion of bacterial taxa and on the properties of their surface glycans to differentially stimulate immune cells.

3 | DISCUSSION

This study showed that the intestinal inflammation occurring in Il10−/− mice is accompanied by changes in carbohydrate hydrolase activities and levels of free monosaccharides in the caecum. Luminal monosaccharides represent nutrients for bacteria able to use these molecules as carbon source. Through the supplementation of caecum bacteria with azido-monosaccharides, we identified selected Bacteroides species as the main bacterial group in Il10−/− mice responding to increased GlcNAc availability by incorporation of the monosaccharide on their surface glycans. The variable responses of specific Bacteroides species to monosaccharide supply and the different effects of Bacteroides species in respect to the activation of dendritic cells underlined the importance to consider bacteria down to the species level when addressing the impact of the microbiota on disease development and progression.

The elevated levels of free monosaccharides detected in Il10−/− mice contrasted with the decreased activity in carbohydrate hydrolases. Considering this lower hydrolase activity, the increased monosaccharide concentrations in the caecum probably reflected a decreased overall consumption of carbohydrates in the inflamed gut of Il10−/− mice. The presence of GalN likely reflected a concomitant increased GalNAc-specific deacetylase activity of bacterial origin in the caecum of Il10−/− mice. The increased occurrence of GalN could also result from the enhanced degradation of intestinal glycosaminoglycans during colitis, which has been shown to be a carbohydrate metabolite entailing direct cytotoxic effects on intestinal cells (Lee, Han, Ryu, & Kim, 2009). Beyond bacterial carbohydrate hydrolases released in the intestinal lumen, host enzymes may also contribute to the increased glycotoxic activity, as recently shown by the induction of NEU3 neuraminidase during intestinal inflammation through TLR4-dependent mechanisms (Yang et al., 2017). Changes in carbohydrate cleavage from the intestinal mucosa likely also alter the adhesion of microbes to the mucus by removing ligands or unmasking new binding structures, thereby enabling changes in the microbiota composition. For example, alteration of mucosal glycans may affect the localisation of lactobacilli that express mucus-binding proteins (Etzold & Juge, 2014). Also, the adhesion of pathogenic bacteria such as Campylobacter jejuni and E. coli K99 relies on adhesins binding to fucosylated structures (Mahdavi et al., 2014) and fimbriae binding to sialylated epitopes (Kyogashima, Ginsburg, & Krivan, 1989), respectively. Increased degradation of mucus glycans may also alter intestinal barrier function, although mucus production in the caecum of Il10−/− mice appeared normal by 15 weeks of age.

Our analysis of GlcNAz uptake in caecum bacteria revealed Xanthomonadaceae and Pseudomonadaceae in WT mice, and Bacteroidaceae in Il10−/− mice as the main bacterial families incorporating this monosaccharide on their surface glycans. Xanthomonadaceae are gram-negative bacteria that express proteins involved in the degradation of N-glycans (Assis et al., 2017). Pseudomonadaceae, like Xanthomonadaceae, belong to the phylum of Proteobacteria. Several members of Pseudomonadaceae express carbohydrate hydrolases, such as PsIg in Pseudomonas aeruginosa, which is important for polysaccharide biofilm formation (Baker et al., 2015). In Il10−/− mice, Bacteroidaceae represented the main family in GlcNAz-incorporating bacteria. Metabolic oligosaccharide engineering and fluorophore labelling have successfully been employed before to demonstrate an incorporation of GalNAz in several commensal species including B. fragilis, B. vulgatus, B. thetaiotaomicron and B. ovatus (Geva-Zatorsky et al., 2015). Bacteroidaceae comprise many mucolytic species, such as B. vulgatus and B. thetaiotaomicron (Tailford, Crost, Kavanaugh, & Juge, 2015). A broader combination of catabolic pathways and carbohydrate hydrolases enables nearly all Bacteroides species detected in the human intestine to utilise at least a third of known mucosal glycan structures (Ravcheev & Thiele, 2017). The incorporation of GlcNAz on Bacteroides species is expected considering the inclusion of GlcNAc in the core of lipid A (Raetz & Whitfield, 2002). ManNac and SiaNac are by contrast only found in the O-antigen region of LPS or on glycoproteins. Some Bacteroides species express enzymes processing N-acetylenuraminic acid through ManNac epimerisation (Brigham et al., 2009), which may account for the incorporation of these monosaccharides on surface glycoconjugates. Little is known about the O-antigen structure of the Bacteroides species investigated. The LPS of B. vulgatus consists of multiple O-antigen repeats (Hashimoto et al., 2002), whereas B. thetaiotaomicron appears to only present a short O-antigen on its LPS (Jacobson, Choudhury, & Fischbach, 2018).

In addition to peptidoglycan, LPS and glycoproteins, several Bacteroides species express capsular polysaccharides (Fletcher, Coyne, Villa, Chatzidakis-Livanis, & Comstock, 2009). Some of these capsular polysaccharides exert immune-regulatory effects, such as mitigating intestinal inflammation (Mazmanian, Round, & Kasper, 2008) and inducing T regulatory cells (Neff et al., 2016). The incorporation of azido-monomosaccharides in surface glycans, such as capsular polysaccharides, enables the labelling of these glycans and their tracking in the context of antigen presentation and immune cell activation. The different patterns of cytokine expression induced by B. acidifaciens and B. vulgatus in dendritic cells underlined the distinct immune-regulatory properties of these related bacterial species. This variation in cell activation and cytokine response probably reflects the binding of bacterial glycans to different C-type lectins on dendritic cells. The analysis of dendritic cell activation and cytokine production induced by B. acidifaciens and B. vulgatus also emphasises the significant differences in the pro-inflammatory effect of these two species.

Whereas azido-monomosaccharides enable the identification of specific bacteria incorporating specific carbohydrates within broad communities, the success of the approach is limited to bacteria that do not cleave the azido group, such as for example by deacetylation. After entering the cell, exogenous GlcNAc is immediately phosphorylated to GlcNac-6-PO₄, enabling the bacterium to distinguish endogenous and exogenous GlcNAc in order to keep the balance between...
Bacteroides vulgatus (DSM 1447 T), 4.2

Mice were of C57BL/6 background. WT and

synthesis and catabolism (Konopka, 2012). GlcNAc-6-PO4 is often

M9 minimal medium (Sambrook & Russel, 2001) for supplementation

appearing proposed for E. coli (Uehara & Park, 2004). Another drawback of

strain EHV2 was cultured and supplemented aerobically at 37

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EXPERIMENTAL PROCEDURES

4.1 | Mouse models

Mice were of C57BL/6 background. WT and Il10−/− (Kuhn et al., 1993) were bred in house for at least six generations and

M9 minimal medium (Sambrook & Russel, 2001) for supplementation

were grown and supplemented in PYG (DSMZ, medium no. 104 without glucose) or chopped meat medium (DSMZ, medium

4.2 | Bacterial species and culture

Bacteroides vulgatus (DSM 1447T), Bacteroides intestinalis (DSM 17393T), Bacteroides thetaiotaomicron (DSM 2079T), Bacteroides

Bacteroides acidificiens (DSM 15896 T), Lactobacillus plantarum subsp. plantarum (DSM 20174T), Lactobacillus brevis (DSM 20054T), Barnesiella

and azide label-negative bacteria was done using a FACSAria III cell

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After supplementation with azido sugars, bacterial cultures were pel- 

After supplementation with azido sugars, bacterial cultures were pel- 

Bacterial DNA was isolated from caecum content and bacterial cul-

4.4 | Analysis of azido sugar incorporation by flow
cytometry

After supplementation with azido sugars, bacterial cultures were pel-

4.5 | 16S rRNA next generation sequencing

Bacterial DNA was isolated from caecum content and bacterial cul-

bacteria were manipulated under CO2 flushing applying Hungate
techniques (Hungate, 1969) and cultivated anaerobically in rubber-

4.3 | Monosaccharide supplementation of murine
microbiota

Euthanized mice were transferred to an anaerobic box (Bugbox M, Baker

were grown and supplemented in PYG (DSMZ, medium no. 104 without glucose), supplemented with 1 mM Glc and 1 mM GlcNAc (Carbosynth,

the approach relates to the short period of labelling with azido-monosaccharides. The resulting transitory picture of GlcNAz uptake by

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2 mM CuSO4, 4 mM Tris[(1-benzyl-488-alkyne dye (Molecular probes®, Thermo Fisher Scientific) using
copper-catalysed click chemistry (2 mM CuSO4, 4 mM Tris[(1-benzyl-

Therefore, the specificity of click reactions was monitored through
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SiaNAz), respectively. The azido sugars were synthesised at Glycom A/S (Hørsholm, Denmark). After incubation under rotation at 37°C for 6 hr, liquid cultures were pelleted and prepared for analysis by flow cytometry or DNA was extracted using the QIAamp Fast DNA stool mini kit (Qiagen) for 16S rRNA sequencing.

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Report of the ethics board.

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bacteria may not reflect the natural incorporation of mucosal carbohydrate by the gut microbiota at steady-state. Slowly growing

were manipulated under CO2 flushing applying Hungate techniques (Hungate, 1969) and cultivated anaerobically in rubber-sealed Hungate tubes at 37°C. Bacteroides and Barnesiella species were grown and supplemented in PYG (DSMZ, medium no. 104 without glucose) or chopped meat medium (DSMZ, medium no. 78 including haemin and vitamin K3). Akkermansia muciniphila was cultured in modified YCFA medium in which vitamins and fatty acids were omitted, but mucin type II was added (8 mg/100 ml). Bacteria were supplemented with 1 mM GlcNAc, GlcNAz, ManNAz or SiaNAz. Cell density was measured by a spectrophotometer at 600 nm (WPA S1200+, Biochrom).

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underwent a proteinase K digest (2% proteinase K in 50 mM KCl, 10 mM Tris Base, pH 8, 0.45% Nonidet P-40, 0.45% Tween 20) for 45 min at 50°C. 16S rRNA was amplified using universal primers 8F and 1492R (Chassard, Goumy, Leclerc, Delhomme, & Bernalier-Donadille, 2007). The 16S rRNA V3-V4 region was amplified in a second PCR using primer V3F 340-356 (5'-CTTCTCCCTACGAGCAGC CTCTTGCGATCT-CCTACGGGAGGCAGCAG-3') and V4R 805-876 (5'-GGA GTT CAG ACG TGT GCT TCT CCG ATC T-GACTACH VGGGTWTCTAAT-3') (Schloss, Junier, Kourmouzas, Westcott, & Highlander, 2016). Both primers contained an Illumina adapter at the 5′-end. Sequencing was performed with the Illumina MiSeq platform (Illumina, San Diego) using v2 chemistry and 250x2 read length. Bioinformatic analysis of 16S sequence was performed as previously described (Appert et al., 2020). Briefly, Illumina adaptors and gene-specific primers were removed using cutadapt (Didion, Martin, & Collins, 2017). Sequences were then processed using the DADA2 pipeline (Callahan et al., 2016), which allows inference of exact amplicon sequence variants (ASVs). Reads pairs were truncated after 231 and 229 nucleotides for forward and reverse reads, respectively. After truncation, reads with expected error rates higher than 4 and 5 for forward and reverse reads were removed. After filtering, error rate learning, ASV inference and denoising, reads were merged with a minimum overlap of 20 bp. Chimeric sequences were identified and removed using “consensus” method and taxonomy assignments were performed using dada2 against SILVA database (v.138) (Glockner et al., 2017). Further analysis was performed using QIIME 1 (Caporaso et al., 2010).

4.6 | Quantitative PCR

*Bacteroides* spp., *B. acidifaciens* and *B. vulgatus* were quantified as proportion of total bacterial 16S rRNA amplicons by real-time PCR using the KAPA SYBR® FAST qPCR Master Mix Kit (Kapa Biosystems). Cycling conditions were 50 cycles at 95°C for 10 s and 60°C for 30 s after an initial denaturation at 95°C for 3 min. Primer pairs specific for *Bacteroides* spp. (forward: 5′-AAAGTCCCCCACCATTGG-3′; reverse: 5′-GAGCCGCAAATCTTACAAA-3′) and total bacteria (515F: 5′-GTGCGAGCMCAGGGTTAAG-3′; 805R: 5′-GACTACAGGGTATAATACAA-3′) were described previously (Frank et al., 2007; Franks et al., 1998; Manz, Amann, Ludwig, Vancanneyt, & Schleifer, 1996). Primer pair specific for the recA gene for recombinae A of *B. acidifaciens* (forward: 5′-AACCTGTAAACGGTGAACAGGCGG-3′; reverse: 5′-GCTGACAGCGGAGTCTAATGGT-3′) was designed. The lack of significant sequence similarity of the selected primers with unrelated bacterial sequences was confirmed by BLAST analysis. The primers for the B. vulgatus sialidase gene Bv-1266 (5′-GGAGGGGAAGACCTTATTTGC-3′) and Bv-r501 (5′-TTCCACC ACTTCTCCGCCGAC-3′) were used as described previously (Huang et al., 2015). The 2^ΔΔCt method was used to calculate quantification values relative to total bacterial 16S rRNA amplicons (Schmittgen & Livak, 2008).

4.7 | Carbohydrate hydrolase activity assays

The caecum content of 8–10 week-old mice was collected and centrifuged at 16,000 × g for 20 min at 4°C. The resulting supernatants were applied for carbohydrate hydrolase assays. Galactosidase, fucosidase, and N-acetylgalcosaminidase activities were measured using the colorimetric substrates 4-nitrophenyl α-D-galactopyranoside (Sigma), 4-nitrophenyl β-D-galactopyranoside (Carbosynth), N-acetyl-β-D-glucosamine (Carbosynth), and 4-nitrophenyl N-acetyl-β-D-glucosamine (Sigma). Sialidase activity was measured using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt (4MU-NeuNAC; Carboxynth). Assays consisting of 10 μl caecum fluid, 50 μl of 6 mM 4-nitrophenyl-carbohydrate substrate and 190 μl 0.1 M phosphate-buffered saline, pH 6.5 were incubated at 37°C for 30 min. Assays were stopped by addition of 250 μl 0.5 M Na₂CO₃, pH 10.5. Cleaved 4-nitrophenol was measured by colorimetric detection in a microplate reader (Tecan, Infinite M200 Pro) via absorbance measurement at a wavelength of 405 nm. For sialidase activity, assays consisted of 10 μl caecum fluid and 190 μl of 0.1 mM 4MU-NeuNAC in 100 mM Tris-Cl buffer, pH 7.4 incubated at 37°C for 15 min. Assays were stopped by addition of 800 μl of 0.5 M Na₂CO₃, pH 10.5. Cleaved 4-MU was measured by fluorescence detection in a microplate reader (Tecan, Infinite M200 Pro) at an excitation wavelength of 360 nm and an emission wavelength of 440 nm.

4.8 | Quantification of monosaccharides

Caecum samples were isolated and processed as for carbohydrate hydrolase activity assays. The supernatant was filtered through a 0.45 μm cronus HPLC membrane and stored at −20°C before use. 5 μl were injected into a Dionex ICS-5000+ ion chromatography system (Thermo Fisher Scientific) and monosaccharides were separated on a PA1 CarboPac column, 250 × 4 mm, with 10 mM NaOH at a flow rate of 1 ml min⁻¹. Fuc, GalN, GlcN, Gal, Glc, GlcNAc and GalNAc were identified and quantified by comparison with authentic standards. For the quantification of sialic acids, samples were derivatised with 1.2-diamino-4,5-methylene- dihydrochloride (DMB, Sigma Aldrich) as described previously (Har et al., 1989). In brief, 3 μl of caecum fluid were incubated with 200 μl of 3.5 mM DMB dihydrochloride in 1.7 M acetic acid containing 375 mM 4-methylumbelliferyl-α-D-neuraminic acid sodium salt (4MU-NeuNAC; Carboxynth). Assays consisting of 10 μl caecum fluid and 190 μl of 0.1 mM 4MU-NeuNAC in 100 mM Tris-Cl buffer, pH 7.4 incubated at 37°C for 2.5 hr in the dark. The reaction was stopped by adding 800 μl of 0.5 M Na₂CO₃, pH 10.5. Cleaved 4-MU was measured by fluorescence detection in a microplate reader (Tecan, Infinite M200 Pro) at an excitation wavelength of 360 nm and an emission wavelength of 440 nm.
4.9 | Stimulation of bone marrow-derived dendritic cells

Bone marrow-derived murine dendritic cells were generated following the protocol of Matheu et al. (Matheu, Sen, Cahalan, & Parker, 2008). The water lysis of the red blood cells was replaced by treatment with ammonium-chloride-potassium (ACK) lysing buffer (150 mM NaCl, 10 mM KHCO3, 0.1 mM EDTA). Mature dendritic cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco) containing 10% FCS, 1 mM sodium pyruvate and 1x GlutaMAX™ supplement (Gibco). Cells were stimulated with fixed bacteria, PBS or LPS for 18 hr at 37°C. Before stimulation, B. acidifaciens and B. vulgatus were grown for 16 hr in PYG supplemented with 5, 10 or 20 mM of either GlcNAc, ManNAc or SiaNAc or PYG medium alone. The density of the supplemented Bacteroides species were normalised by OD600 measurement and cell counting by cytometry. Equal bacterial counts were fixed in 2% paraformaldehyde for 15 min at room temperature, washed with PBS and co-cultured with dendritic cells in a ratio of 10:1 for 18 hr. Stimulation with O111:B4 E. coli LPS was done at a concentration of 100 ng/ml. After stimulation, dendritic cells were stained with fluorochrome-labelled anti-mouse antibodies CD40-APC, CD86-PE and MHC-II-FITC (BioLegend) for 30 min on ice. Cells were analysed using a FACSCanto II flow cytometer (BD Bioscience). Cytokine expression after stimulation was determined by ProcartaPlex™ Multiplex immunoassay (ThermoFisher Scientific) according to the manufacturer’s instructions.

4.10 | Isolation and analysis of LPS

LPS was isolated as described by Davis and Goldberg (Davis Jr. & Goldberg, 2012) with minor modifications. In brief, cultures were washed twice with PBS, OD600 was measured and cultures diluted to reach equal cell densities. Bacteria were centrifuged at 12,000 x g for 10 min and cell pellets were lysed by boiling in 600 μl of SDS buffer consisting of 2% SDS, 10% glycerol, 2% β-mercaptoethanol in 50 mM Tris–HCl, pH 6.8 for 15 min. After cooling on ice, 2 mg of DNase I and RNase A were added and samples were incubated for 30 min at 37°C, then 2 mg proteinase K was added and samples were further incubated overnight at 59°C. Phenol (600 μl) was added, samples were incubated at 65°C for 15 min after vortexing. After cooling on ice, 3 ml of diethylether was added and samples vortexed and spun at 10,000 x g for 10 min. The lower aqueous phase was extracted and the phase separation with phenol and diethylether was repeated once. To each sample, 500 μl of 2X SDS buffer was added prior to performing gel electrophoresis in 14% polyacrylamide gels (Davis Jr. & Goldberg, 2012). LPS was developed by silver staining following the procedure of Fomsgaard et al. (Fomsgaard, Freudenberg, & Galanos, 1990).

ACKNOWLEDGEMENTS

We thank Annelies Geirnaert (ETH-Zurich) for her assistance with the bioinformatic analysis of 16S rRNA sequencing data. This work was supported by the Swiss National Foundation grant CRSII5_180353 to CL and TH, and grant 314730_172880 to TH.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Gisela Adrienne Weiss and Thierry Hennet designed the study. Nikolay Khanzin synthesised the azido sugars, Gisela Adrienne Weiss and Tobias Hasler analysed caecum monosaccharides and carbohydrate hydrolase activities, Thomas Grabinger isolated LPS and performed dendritic cells assays with Gisela Adrienne Weiss and Tobias Hasler. Gisela Adrienne Weiss and Jesús Glaus Garzon performed FACS-based bacterial sorting. Gisela Adrienne Weiss, Anna Greppi and Christophe Lacroix analysed 16S rRNA sequencing data. Gisela Adrienne Weiss, Thomas Grabinger and Thierry Hennet wrote the manuscript. All authors contributed to the revision of the manuscript. Thierry Hennet secured the funding.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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