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# *Akkermansia muciniphila* upregulates genes involved in maintaining the intestinal barrier function via ADP-heptose-dependent activation of the ALPK1/TIFA pathway

Camille Martin-Gallausiaux <sup>a</sup>, Diego Garcia-Weber <sup>b</sup>, Amandine Lashermes<sup>a</sup>, Pierre Larraufie <sup>a</sup>, Ludovica Marinelli <sup>a</sup>, Veronica Teixeira<sup>b</sup>, Alice Rolland<sup>a</sup>, Fabienne Béguet-Crespel<sup>a</sup>, Vincent Brochard<sup>a</sup>, Timothé Quatremare<sup>a</sup>, Alexandre Jamet<sup>a</sup>, Joël Doré <sup>a</sup>, Scott D. Gray-Owen <sup>c</sup>, Hervé M. Blottière <sup>a</sup>, Cécile Arriemerlou <sup>b\*</sup>, and Nicolas Lapaque <sup>a\*</sup>

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## ABSTRACT

The commensal bacteria that make up the gut microbiota impact the health of their host on multiple levels. In particular, the interactions taking place between the microbe-associated molecule patterns (MAMPs) and pattern recognition receptors (PRRs), expressed by intestinal epithelial cells (IECs), are crucial for maintaining intestinal homeostasis. While numerous studies showed that TLRs and NLRs are involved in the control of gut homeostasis by commensal bacteria, the role of additional innate immune receptors remains unclear. Here, we seek for novel MAMP-PRR interactions involved in the beneficial effect of the commensal bacterium *Akkermansia muciniphila* on intestinal homeostasis. We show that *A. muciniphila* strongly activates NF- $\kappa$ B in IECs by releasing one or more potent activating metabolites into the microenvironment. By using drugs, chemical and gene-editing tools, we found that the released metabolite(s) enter(s) epithelial cells and activate(s) NF- $\kappa$ B via an ALPK1, TIFA and TRAF6-dependent pathway. Furthermore, we show that the released molecule has the biological characteristics of the ALPK1 ligand ADP-heptose. Finally, we show that *A. muciniphila* induces the expression of the *MUC2*, *BIRC3* and *TNFAIP3* genes involved in the maintenance of the intestinal barrier function and that this process is dependent on TIFA. Altogether, our data strongly suggest that the commensal *A. muciniphila* promotes intestinal homeostasis by activating the ALPK1/TIFA/TRAF6 axis, an innate immune pathway exclusively described so far in the context of Gram-negative bacterial infections.

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## Introduction


The host gastrointestinal tract is colonized by a complex microbial community, referred as gut microbiota. It is well established that, through the production of microbial metabolites, commensal bacteria perform many biological functions, which have a direct impact on the health of the host.<sup>1</sup> They favor the clearance of pathogens and the repair of damaged tissues, increase barrier functions, promote the differentiation and proliferation of intestinal epithelial cells (IECs) as well as the development and maturation of the sub-epithelial immune cells.<sup>2–4</sup> The maintenance of intestinal homeostasis depends on a tightly regulated crosstalk between the gut microbiota, IECs and mucosal immune cells.<sup>4</sup> It

is initiated through the activation of innate immune receptors, also called pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs). PRRs recognize a wide range of ligands named microbe-associated molecular patterns (MAMPs) and trigger complex downstream signaling pathways that converge to the regulation of important transcription factors like NF- $\kappa$ B.<sup>2,3,5</sup> Among commensals, the Gram-negative bacterium, *Akkermansia muciniphila*, emerged as one of the key players of the microbiota by having a positive impact on host health, notably on obesity and colitis disorders.<sup>6,7</sup> This is supported by findings showing that administration of *A. muciniphila* has

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beneficial effects on metabolic and inflammatory responses in mouse models with similar associations in human.<sup>7–10</sup> Indeed, administration of *A. muciniphila* reduces weight gain and improves host metabolic parameters such as insulin resistance and glucose tolerance in high-fat diet models.<sup>7</sup> Moreover, this bacterium improves clinical parameters in DSS-induced colitis.<sup>10</sup> Such beneficial properties have been associated with the strengthening of gut barrier functions induced by *A. muciniphila* that counteract high-fat diet-induced endotoxemia or colitis-induced inflammation.

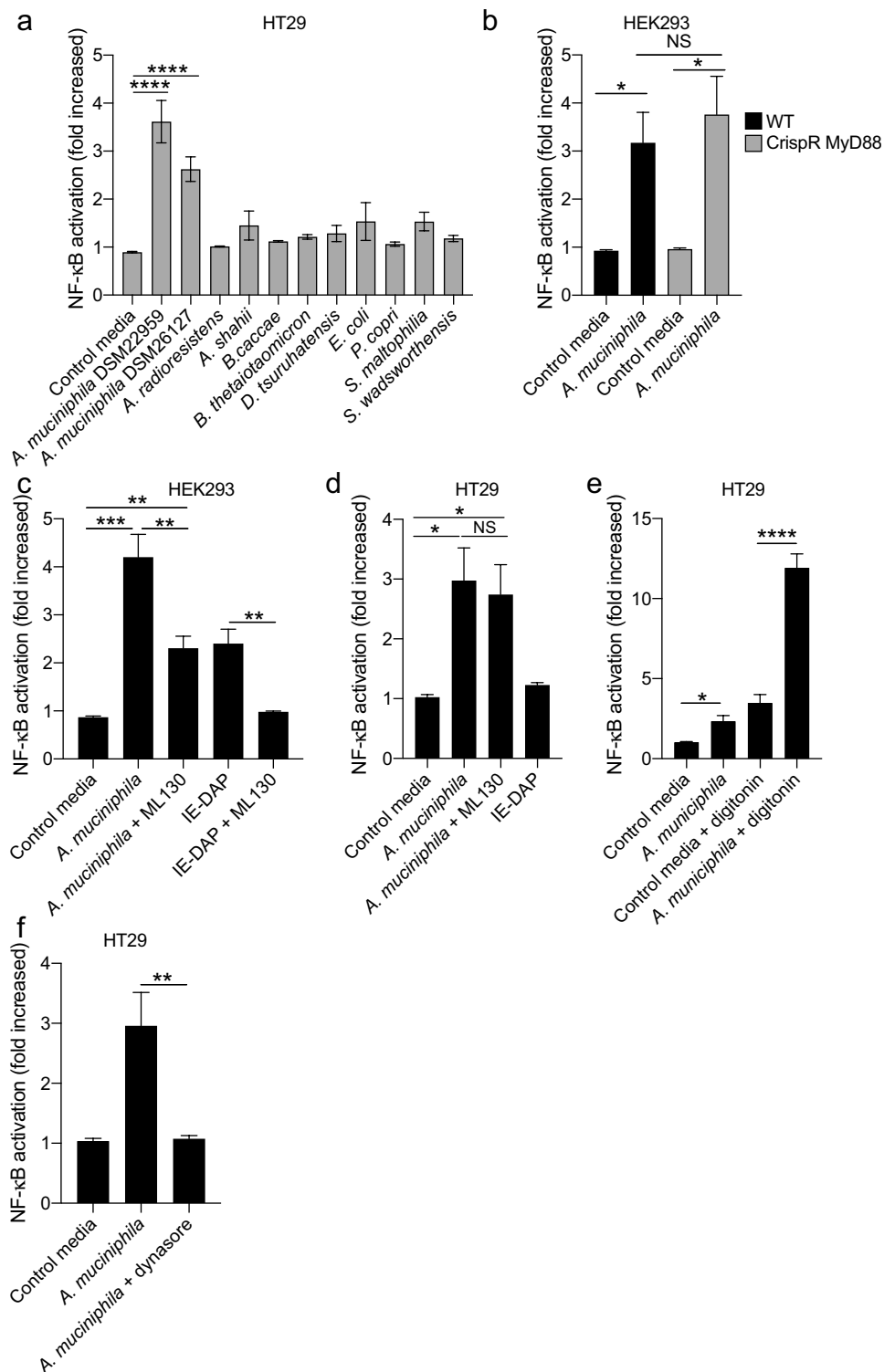
Recently, alpha kinase 1 (ALPK1) has been described as a new PRR sensing ADP-heptose (ADP-H), a soluble intermediate of the lipopolysaccharide (LPS) biosynthetic pathway present in most Gram-negative bacteria.<sup>11–13</sup> In response to ADP-H binding on its N-terminal domain, ALPK1 phosphorylates TIFA proteins on their threonine 9 residue. This mechanism induces the formation of large TIFA oligomers named TIFAsomes, and the downstream activation of NF- $\kappa$ B in a TRAF6-dependent manner. ALPK1-mediated ADP-H sensing occurs during infection by several bacterial pathogens, including *Shigella flexneri*, *Yersinia pseudotuberculosis*, *Helicobacter pylori* and *Campylobacter jejuni* confirming a broad involvement of this new pathway of innate immunity in Gram-negative bacterial infections.<sup>11,12,14–16</sup> Interestingly, ALPK1 regulates intestinal homeostasis in mouse models of pathogen-induced colitis<sup>11</sup> but the potential activation of this pathway by gut microbiota has not been investigated yet.

Here, we show that *A. muciniphila* can activate NF- $\kappa$ B in IECs independently of TLRs and NOD receptors, and this process is mediated by the release of a soluble metabolite into its microenvironment. This metabolite activates NF- $\kappa$ B in an ALPK1, TIFA and TRAF6-dependent manner and has the biological characteristics of ADP-H. Moreover, *A. muciniphila* promotes the expression of genes involved in the maintenance of intestinal barrier function via the activation of the ALPK1/TIFA axis, suggesting that this pathway contributes to the beneficial impact of this bacterium in intestinal homeostasis.

## Results

### **A. muciniphila releases a metabolite that induces NF- $\kappa$ B activity independently of TLRs and NOD1 in IECs**

In order to characterize new underlying molecular mechanisms responsible for the beneficial impact of the commensal bacterium *A. muciniphila* on the maintenance of the intestinal barrier, we investigated the pathways involved in the activation of NF- $\kappa$ B by this bacterium. For this, we tested the ability of bacterial culture supernatants to activate NF- $\kappa$ B in an NF- $\kappa$ B reporter system expressed in the IEC line, HT29. We found that culture supernatants from two *A. muciniphila* strains of human (DSM22959) and mice (DSM26127) origin increased the NF- $\kappa$ B activity with a >2 fold increase whereas a non-inoculate growth medium had no effect (Figure 1a). This result is specific to *A. muciniphila* strains as supernatants of a selection of intestinal Gram negative commensal bacteria, including members of the Bacteroidetes (*Alistipes shahii*, *Bacteroides caccae*, *Bacteroides thetaiotaomicron* and *Prevotella copri*) and Proteobacteria (*Acinetobacter radioresistens*, *Delftia tsuruhatensis*, *Escherichia coli*, *Stenotrophomonas maltophilia* and *Sutterella wadsworthensis*) phyla, failed to activate NF- $\kappa$ B (Figure 1a). HT29 cells express several TLRs and NF- $\kappa$ B is induced by high concentrations of TLR3, TLR4 and TLR5 ligands.<sup>17</sup> To first determine whether TLRs were responsible for the NF- $\kappa$ B response to *A. muciniphila*, we took advantage of a HEK293 NF- $\kappa$ B-reporter cell system that lacks most endogenous TLRs<sup>18</sup> and showed that *A. muciniphila* culture supernatant also induced a high NF- $\kappa$ B response in HEK293 cells (Figure 1b). To further rule out the implication of TLRs, we implemented the CRISPR/Cas9 gene editing tool to knock down the adaptor protein MYD88, which is involved in several TLR signaling pathways.<sup>19</sup> Using this approach, we generated a *MYD88* knockout (*MYD88*<sup>-/-</sup>) clone derived from the HEK-NF- $\kappa$ B reporter cell and assessed the invalidation of the gene by monitoring the MYD88 protein by western-blot (supplementary Figure S1B). WT and *MYD88*<sup>-/-</sup> HEK cells were then incubated for 24 h with an *A. muciniphila* culture supernatant. Data showed that NF- $\kappa$ B was similarly activated in both cell



**Figure 1.** *A. muciniphila* supernatants activate NF-κB in IECs via a pathway that does not involve MYD88 and NOD1. A. Average NF-κB activity in HT29-NFκB reporter system induced by a wide range of supernatants derived from commensal Gram negative bacteria. B. HEK-NF-κB reporter (WT, black bars) and deleted for MYD88 (MYD88<sup>-/-</sup>, gray bars) cells were incubated with *A. muciniphila* DSM22959 supernatant or control media for 24 h. C-D Treatment of HEK-NF-κB (c) or HT29-NF-κB reporter cells with control media, *A. muciniphila* DSM22959 supernatant or the NOD1 ligand, IE-DAP for 24 h in presence or absence of a NOD1 inhibitor, ML130. E. NF-κB activity in HT29 cells treated with *A. muciniphila* DSM22959 supernatant in the presence or absence of the permeabilizing agent digitonin for 30 min. When incubated with digitonin, cells were washed with fresh media before measuring the NF-κB activity at 24 h. F. NF-κB activity in HT29 cells pretreated with DMSO or dynasore for 1 h prior to treatment with *A. muciniphila* supernatant. NF-κB activation was measured by SEAP secretion and expressed as mean ± SEM fold change toward unstimulated cells. Data represent ≥3 independent experiments performed in duplicate or triplicate. Data analysis: unpaired t test was used, \*\*\*\*P < 0,0001; \*\*\*P < 0,001; \*\*P < 0,01; \*P < 0,05; P > 0,05 was considered as not significant (ns).

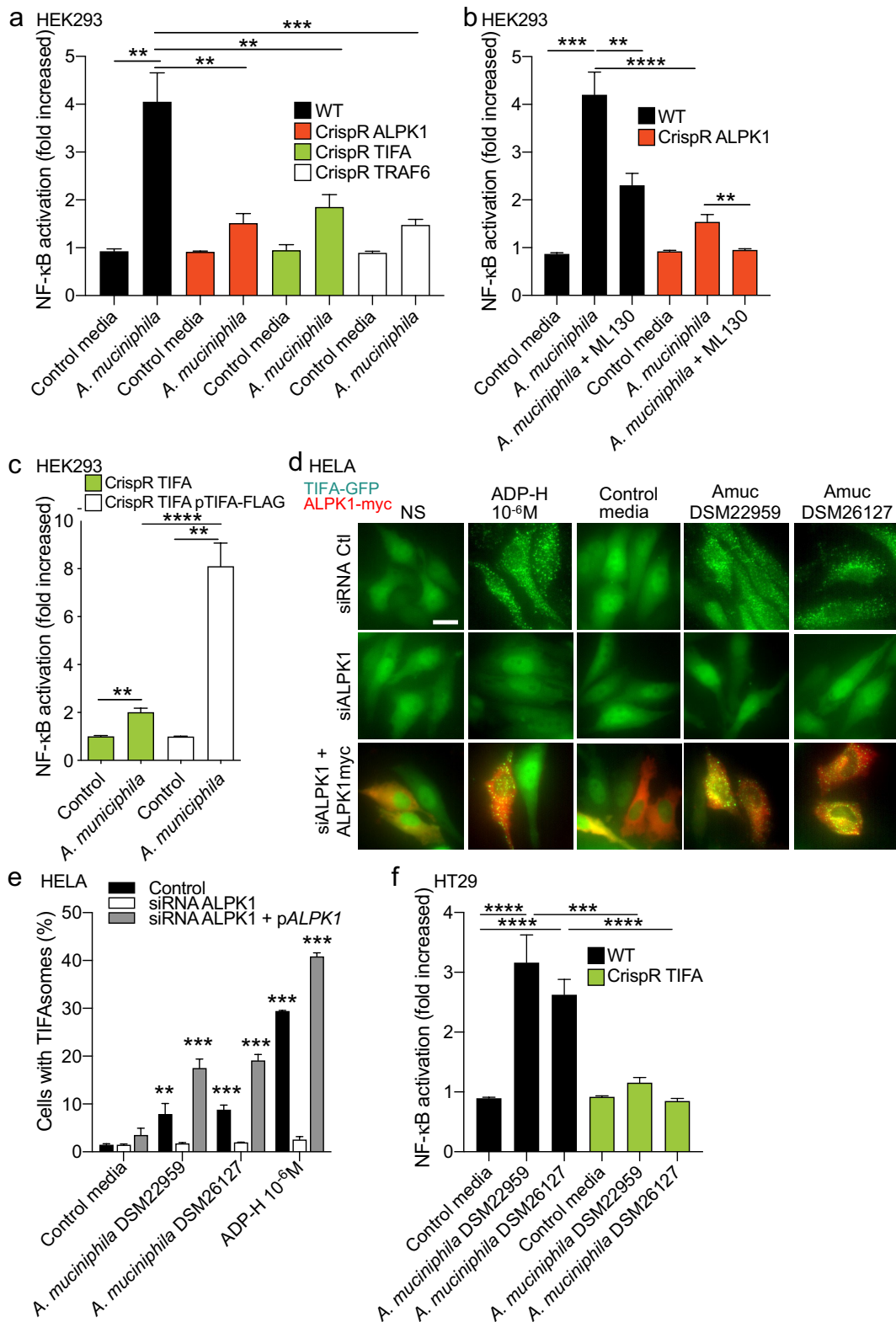
lines, excluding a role of MYD88-dependent TLRs in *A. muciniphila*-induced NF- $\kappa$ B activation (Figure 1b). Altogether, our results strongly suggest that this commensal bacterium activates NF- $\kappa$ B independently of TLRs.

NOD1/2 receptors can also induce NF- $\kappa$ B in response to commensal bacteria independently of MYD88/TLR.<sup>20</sup> As HEK293 cells do not express NOD2,<sup>21,22</sup> we ruled out the implication of this PRR in *A. muciniphila*-induced NF- $\kappa$ B activation. By treating HEK293 cells with ML130, a specific inhibitor of NOD1,<sup>23</sup> we showed that this PRR was only partially involved in *A. muciniphila*-dependent activation of the NF- $\kappa$ B pathway in these cells (Figure 1c). As expected, we found that ML130 completely abolished the activation of NF- $\kappa$ B induced by the NOD1 ligand IE-DAP. In HT29 cells, ML130 had no effect on *A. muciniphila*-induced NF- $\kappa$ B activation (Figure 1d), showing that the NOD1 pathway was not involved in this process in this intestinal epithelial cell line. To further characterize the molecular pathway implicated in *A. muciniphila*-induced NF- $\kappa$ B activation, we tested whether it required a mechanism of intracellular sensing. For this, we bypassed a potential process of cellular internalization by measuring NF- $\kappa$ B activity in cells that were semi-permeabilized with digitonin. Data showed that digitonin pre-treatment strongly increased *A. muciniphila*-induced NF- $\kappa$ B activation (Figure 1e), indicating that direct access to the cytoplasm was critical and that a mechanism of intracellular sensing was thus required for robust activation of the pathway. In line with this, we used dynasore that blocks dynamin-mediated endocytosis.<sup>24</sup> In HT29 cells pre-incubated with this inhibitor, the NF- $\kappa$ B activity induced by *A. muciniphila* supernatant was decreased (figure 1f). Altogether, our results suggest that *A. muciniphila* activates the NF- $\kappa$ B signaling pathway in IECs through a cytosolic receptor different from TLRs and NOD1/2.

### **In IECs, *A. muciniphila* activates NF- $\kappa$ B via the ALPK1/TIFA/TRAF6 pathway.**

In view of the recent discovery of ALPK1 as an important cytosolic receptor in the innate immune response to Gram-negative pathogens, we

hypothesized that ALPK1 was involved in *A. muciniphila*-induced NF- $\kappa$ B activation in IECs. In response to ADP-H binding, ALPK1 phosphorylates TIFA on threonine 9. This process triggers the oligomerization of TIFA and TRAF6 and the downstream activation of NF- $\kappa$ B.<sup>11,13</sup> Using CRISPR/CAS9 approaches, we knocked-down *ALPK1*, *TIFA* and *TRAF6* genes in HEK293 cells bearing an NF- $\kappa$ B reporter system (Supplementary Figure S1) and assessed NF- $\kappa$ B activation following treatment with *A. muciniphila* supernatants or control medium for 24 hours. NF- $\kappa$ B activation was highly reduced in *ALPK1*<sup>-/-</sup>, *TIFA*<sup>-/-</sup> and *TRAF6*<sup>-/-</sup> cells (Figure 2a). As we showed that NOD1 was partly involved in NF- $\kappa$ B activation in HEK293 cells (Figure 1c), we treated *ALPK1*<sup>-/-</sup> cells with ML130 and found that remaining NF- $\kappa$ B activation was totally abrogated compared to untreated cells (Figure 2b). As expected, *A. muciniphila*-induced NF- $\kappa$ B activation was restored in *TIFA*<sup>-/-</sup> cells ectopically expressing TIFA from a cDNA construct (Figure 2c). Together, these results demonstrated that the ALPK1/TIFA/TRAF6 pathway was involved in *A. muciniphila*-induced NF- $\kappa$ B activation. As TIFA oligomerization rapidly occurs downstream of ALPK1 activation, we analyzed the effect of *A. muciniphila* culture supernatants on TIFAsome formation and investigated the role of ALPK1 in this process. For this, HeLa cells stably expressing GFP-tagged TIFA (TIFA-GFP) were transfected with control or ALPK1-targeting siRNAs for gene silencing. In addition, ALPK1-depleted cells were transfected or not with an ALPK1 cDNA construct for a rescue experiment.<sup>11</sup> In this experimental set-up, 30 minutes of incubation with culture supernatants of *A. muciniphila* of human and mouse origin or synthetic ADP-H induced clear formation of TIFAsomes (Figure 2d and 2e). In all conditions, this mechanism was abolished in ALPK1-depleted cells while TIFAsome formation was restored upon rescued *ALPK1* expression (Figure 2d and 2e). In addition, using CRISPR/CAS9, we showed that *A. muciniphila*-induced NF- $\kappa$ B activation was also abrogated in HT29 *TIFA*<sup>-/-</sup> cells (figure 2f). Altogether, our results strongly support the role of the ALPK1/TIFA pathway in the activation of NF- $\kappa$ B by *A. muciniphila* culture supernatants.



**Figure 2.** *A. muciniphila* supernatant activates the ALPK1-TIFA-TRAF6 axis. A. WT (black bars),  $ALPK1^{-/-}$  (red bars),  $TIFA^{-/-}$  (green bars) and  $TRAF6^{-/-}$  (white bars) HEK-NF- $\kappa$ B reporter cells were stimulated with *A. muciniphila* DSM22959 supernatant or control media for 24 h. NF- $\kappa$ B activation was measured by SEAP secretion and expressed as mean  $\pm$  SEM fold change toward unstimulated cells. B. WT (black bars) and  $ALPK1^{-/-}$  (red bars) HEK-NF- $\kappa$ B reporter cells were incubated with control media, *A. muciniphila* DSM22959 supernatant or *A. muciniphila* supernatant in presence of a NOD1 inhibitor (ML130) for 24 h prior NF- $\kappa$ B activation measurement. C. NF- $\kappa$ B activity was measured in HEK293  $TIFA^{-/-}$  cells transfected (white bars) or not (green bars) with pTIFA and stimulated with *A. muciniphila* DSM22959 supernatant or control media for 24 h. NF- $\kappa$ B activation was measured by SEAP secretion and expressed

### The NF- $\kappa$ B-activating molecule derived from *A. muciniphila* has the biological properties of the ALPK1 ligand ADP-H.

ADP-H is the bacterial MAMP of Gram-negative bacteria that activates NF- $\kappa$ B *via* the ALPK1/TIFA pathway.<sup>11,12</sup> Most of Gram-negative bacteria, including *E. coli*, produce this cytosolic intermediate of the heptose biosynthesis pathway involved in LPS synthesis.<sup>12</sup> Analysis of the KEGG database shows that the genes encoding the enzymes GmhA, HldE and GmhB involved in this pathway, are present in the genome of *A. muciniphila* (Figure 3a and Supplementary Figure S2). HldE is the enzyme producing ADP-H. In *E. coli*, it is a bifunctional enzyme having both heptokinase and ADP-transferase activities.<sup>12</sup> As no genetic tool exists for *A. muciniphila*, in order to determine whether HldE is functional in *A. muciniphila*, we used an *E. coli* expression system where we expressed *A. muciniphila hldE* from the pBAD plasmid (pBAD-hldE) in an *hldE* deletion mutant of *E. coli* ( $\Delta hldE$ ). ADP-H is not secreted by *E. coli*, so bacterial lysates were used to stimulate the HT29-NF- $\kappa$ B reporter cell-line. We found that only lysates from WT or  $\Delta hldE$  bacteria expressing *A. muciniphila hldE* potently activated NF- $\kappa$ B in HT29 (Figure 3b), confirming that the *A. muciniphila* HldE enzyme was functional. To test if this mechanism of NF- $\kappa$ B activation was mediated by the ALPK1-TIFA pathway, we treated HT29 TIFA<sup>-/-</sup> cells with bacterial lysates from WT,  $\Delta hldE$  and  $\Delta hldE$  phldE-expressing *E. coli*. As expected, we found that the activation of NF- $\kappa$ B was completely TIFA-dependent (Figure 3b). Altogether, these results showed that the expression *A. muciniphila hldE* in *E. coli* is sufficient to activate NF- $\kappa$ B in a TIFA-dependent manner. They also strongly suggested that ADP-H, the MAMP that activates the ALPK1/TIFA pathway, was

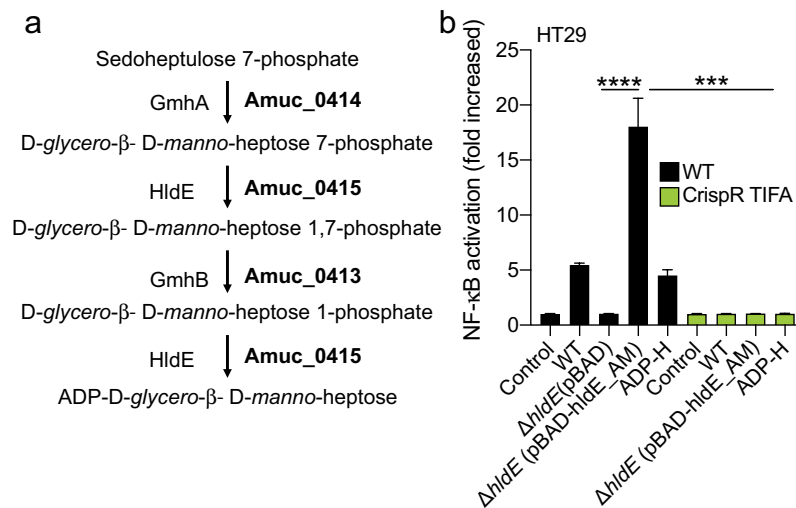
responsible for *A. muciniphila*-induced NF- $\kappa$ B activation. To directly test this hypothesis, we determined the contribution of heptose-1,7-bisphosphate (HBP), the other metabolite produced by HldE, that can indirectly and weakly activate the ALPK1/TIFA pathway after being processed intracellularly by host adenylyltransferases into ADP-H 7-P.<sup>12</sup> For this, we took advantage of the fact that in contrast to ADP-H, HBP is unable to induce the formation of TIFAsomes after a short treatment.<sup>11</sup> We thus monitored the formation of TIFAsomes in TIFA-GFP expressing HeLa cells after 30 minutes. Whereas supernatants from both *A. muciniphila* strains and synthetic ADP-H induced the formation of TIFAsomes within this time period, we confirmed that synthetic HBP failed to do so (Figure 4a and 4b). This result indicated that the kinetics of TIFAsome formation induced by *A. muciniphila* was consistent with that of ADP-H but not with that of HBP (Figure 4a and 4b). ADP-H is a molecule partly resistant to calf intestinal alkaline phosphatase (CIP) but sensitive to the phosphodiesterase (PDE) from *C. adamanteus*.<sup>16</sup> In contrast, HBP is only sensitive to CIP treatment.<sup>16</sup> Therefore, to further exclude a role of HBP in *A. muciniphila*-induced NF- $\kappa$ B activation, we analyzed the effects of treating bacterial culture supernatants with CIP and PDE. Data showed that the *A. muciniphila*-derived NF- $\kappa$ B-activating molecule was partly resistant to CIP and completely sensitive to PDE (Figure 4c). Altogether, these data showed that ADP-H, and not HBP, was responsible for the ALPK1-dependent activation of NF- $\kappa$ B by *A. muciniphila*.

In our different reporter systems, we observed that ADP-H activates NF- $\kappa$ B at concentrations as low as 50–100 nM (Supplementary Figure S3). To precisely quantify the concentration of ADP-H in the supernatant of *A. muciniphila* cultures,

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as mean  $\pm$  SEM fold change toward un-stimulated cells. D-E) After siRNA treatment with Control siRNA (black bars) or ALPK1 specific siRNA (white and gray bars), HeLa cells were transfected with empty pCMV or pCMV-ALPK1-myc and were left unstimulated (NS) or stimulated with the control media, *A. muciniphila* supernatants or ADP-H ( $10^{-6}$ M). D. Representative pictures of cells with TIFAsomes at 30 min in TIFA-GFP-expressing HeLa cells, Scale bar: 20  $\mu$ m. E. The graph shows the quantification of cells with TIFAsomes in each condition as shown in D. Data correspond to the mean  $\pm$  SD from 3 independent experiments performed in triplicate wells. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test \*\*P < 0,01; \*\*\*P < 0,001; \*\*\*\*P < 0,0001. F. WT (black bars) and TIFA<sup>-/-</sup> (green bars) HT29-NF- $\kappa$ B reporter cells were stimulated with *A. muciniphila* supernatants or control media for 24 h. NF- $\kappa$ B activation was measured by SEAP secretion and expressed as mean  $\pm$  SEM fold change toward unstimulated cells. Data represent  $\geq 3$  independent experiments performed in triplicate. Data analysis: unpaired t test was used, \*\*\*\*P < 0,0001; \*\*\*P < 0,001; \*\*P < 0,01; \*P < 0,05; P > 0,05 was considered as not significant (NS).





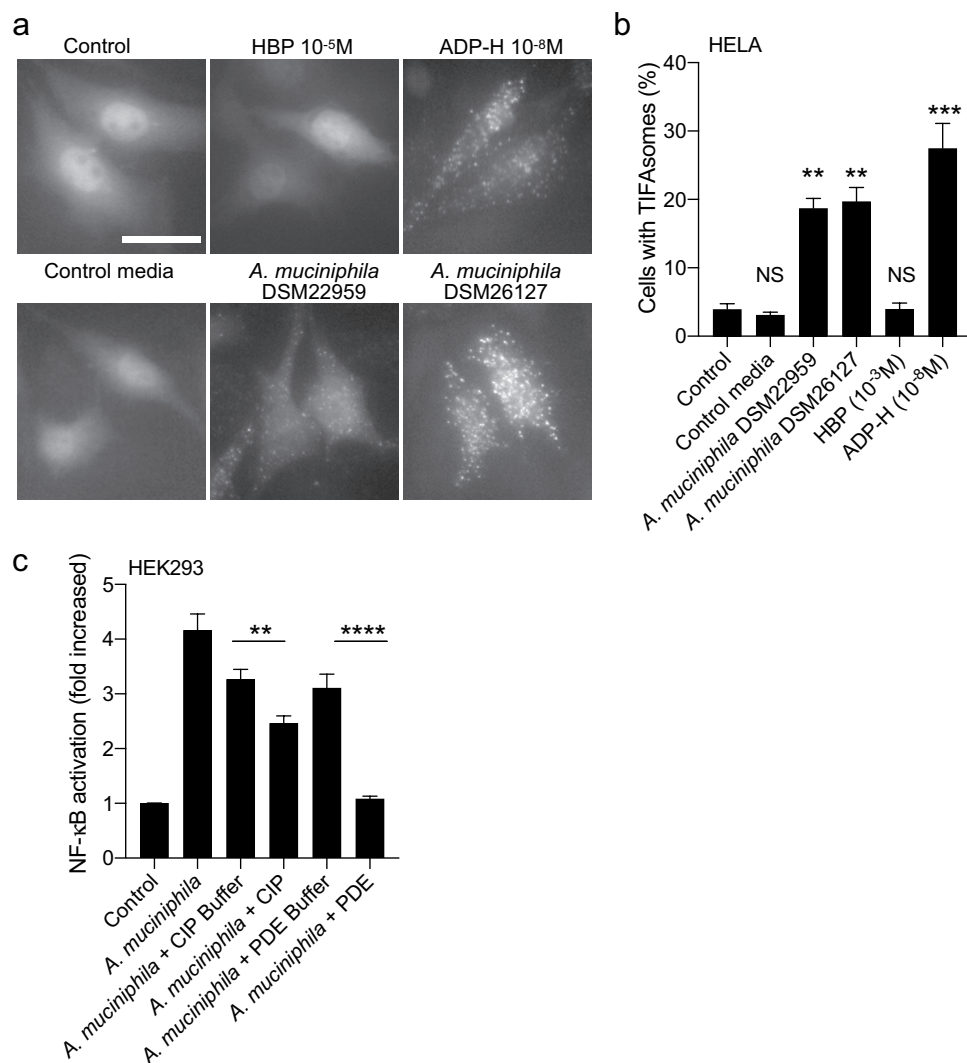
**Figure 3.** *HldE* (Amuc\_0415) from *A. muciniphila* from the heptose biosynthesis pathway is functional. A. Schematic view of the heptose biosynthesis pathway. The gene names annotated for *A. muciniphila* are in bold. B. HT29-NF-κB reporter WT or *TIFA*<sup>-/-</sup> cells were stimulated for 24 h with lysats from *E. coli*; *E. coli* Δ*hldE*; *E. coli* Δ*hldE* transformed with plasmid controls (pBAD), with *hldE* (Amuc\_0415) from *A. muciniphila* (pBAD-*hldE*\_AM) or ADP-H (10<sup>-6</sup> M). NF-κB activation was measured by SEAP secretion and expressed as mean (%) ± SEM fold change toward supernatant-stimulated cells. Data represent ≥3 independent experiments performed in triplicate. Data analysis: unpaired t test was used, \*\*\*\*P < 0,0001; \*\*\*P < 0,001; \*\*P < 0,01; \*P < 0,05; P > 0,05 was considered as not significant (NS).

calibration standard solutions from 10<sup>-8</sup> to 10<sup>-6</sup> M of ADP-H were used in both HT29-NF-κB and HEK-NF-κB reporter cell lines (Supplementary Figure S3A and S3B). We found that the concentration of ADP-H in the *A. muciniphila* supernatant was in the 100 nM range (Supplementary Figure S3C). This is consistent with concentrations produced by pathogens such as *H. pylori*.<sup>16</sup>

### ***A. muciniphila* upregulates genes involved in maintaining the intestinal barrier function via the ALPK1/TIFA pathway**

*A. muciniphila* has a positive impact on the health of the host, notably by promoting the integrity of the intestinal barrier.<sup>8,25</sup> Therefore, we hypothesized that the effect of this commensal bacterium may be mediated by ADP-H-dependent activation of the ALPK1/TIFA pathway and the regulation of genes involved in the maintenance of intestinal homeostasis. Analysis of human transcriptomic datasets showed that *ALPK1* and *TIFA* are widely expressed in intestinal epithelial cell types from the small and large intestines including intestinal stem cells (ISC) and differentiated IECs with *ALPK1* being most expressed in goblet and Paneth cells and less expressed in enteroendocrine cells (Figure 5a and 5b).<sup>26</sup> To determine whether the

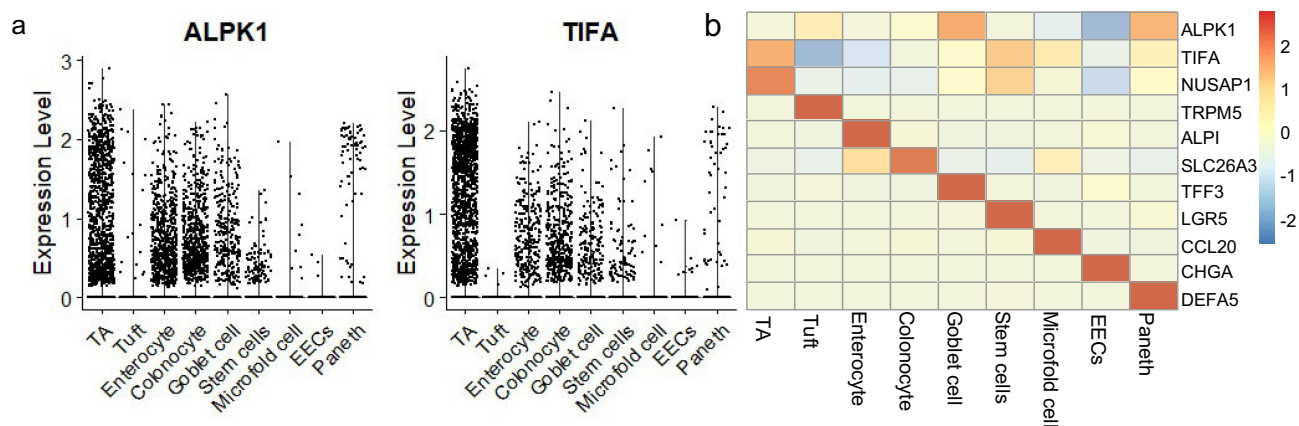
beneficial effect of *A. muciniphila* on the intestinal barrier can be mediated by the ALPK1/TIFA pathway, we analyzed the impact of *A. muciniphila* on the regulation of *MUC2*, *CLAUDIN-3*, *OCCLUDIN*, *BIRC3* and *TNFAIP3*, five genes involved in the maintenance of the intestinal barrier function, and investigated the role of TIFA in this process (Figure 6a-6e). WT and *TIFA*<sup>-/-</sup> HT29 cells were incubated for 6 hours with an *A. muciniphila* culture supernatant, a non-inoculated control medium or ADP-H prior to RNA extraction and RTqPCR. Data revealed a significant up-regulation of *MUC2*, *BIRC3* and *TNFAIP3* expression induced both by *A. muciniphila* supernatant and ADP-H, and that this enhanced expression was TIFA-dependent (Figure 6a-6e). The ALPK1/TIFA axis has also been associated with immune response induction.<sup>13,15</sup> We thus assessed the proinflammatory cytokines *CXCL8*, *IL-1β* and the anti-inflammatory cytokine *TGFβ1* in the same set-up. WT and *TIFA*<sup>-/-</sup> HT29 cells were incubated with an *A. muciniphila* culture supernatant, a non-inoculated control medium or ADP-H for 6 hours. *A. muciniphila* supernatant and ADP-H induced a significant up-regulation of *CXCL8* expression in a TIFA-dependent manner while expression of *IL-1β* and *TGFβ1* was not affected



**Figure 4.** *A. muciniphila* derived molecule activating NF- $\kappa$ B has the biological properties of ADP-H. A. Representative pictures of cells with TIFAsomes at 30 min in TIFA-GFP-expressing HeLa cells. Cells were treated with digitonin and *A. muciniphila* DSM22959 or DSM26127 supernatants, HBP ( $10^{-5}M$ ) or ADP-H ( $10^{-8}M$ ). Scale bar: 20  $\mu$ m. B. Quantification of cells with TIFAsomes in each condition as shown in A. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test \*\* $P < 0,01$ ; \*\*\*\* $P < 0,0001$ ; NS: non-significant. C. *A. muciniphila* supernatants were untreated, treated with calf intestine alkaline phosphatase (CIP), with *Crotalus adamanteus* phosphodiesterase (PDE) or their respective buffer (CIP buffer and PDE buffer) prior to stimulation of HT29-NF- $\kappa$ B reporter cells for 24 h. NF- $\kappa$ B activation was measured by SEAP secretion and expressed as mean (%)  $\pm$  SEM fold change toward supernatant-stimulated cells. Data represent  $\geq 3$  independent experiments performed in triplicate. Data analysis: unpaired t test was used, \*\*\*\* $P < 0,0001$ ; \*\*\* $P < 0,001$ ; \*\* $P < 0,01$ ; \* $P < 0,05$ ;  $P > 0,05$  was considered as not significant (NS).

(figure 6f-6h). In addition, we measured by ELISA the concentration of IL-8 (encoded by *CXCL8*) in HT29 cells and showed that both *A. muciniphila* supernatant and ADP-H induced the secretion of this cytokine in a TIFA-dependent manner (Figure 6i). Several studies reported that *A. muciniphila* improves intestinal barrier integrity.<sup>27-29</sup> We thus evaluated the barrier functions by measuring the transepithelial electrical resistance (TEER) in HT29-Cl.16E cells in presence

of ADP-H. HT29-Cl.16E cells were chosen as they form polarized monolayers, functional tight junctions, secrete mucins<sup>30,31</sup> and importantly, unlike Caco2 cells, express *ALPK1* (Supplementary Figure S4). Interestingly, daily TEER measurement showed that ADP-H improved HT29-Cl.16E monolayer integrity (Figure 7). Altogether these results show that *A. muciniphila* upregulates genes involved in maintaining the integrity of intestinal barrier in a TIFA-dependent manner and that



**Figure 5.** *ALPK1* and *TIFA* are expressed by IECs in human gut. A: *ALPK1* and *TIFA* expression in single cells classified by main epithelial cell types. B: Heatmap of relative average expression of *ALPK1*, *TIFA* and main intestinal epithelial cell markers across intestinal epithelial cell types (TA = Transient amplifying cells; EECs = Enteroendocrine cells). Data was obtained from Elmentaite *et al.*<sup>26</sup> looking at human adult intestinal epithelial cells (<https://www.gutcellatlas.org/>)

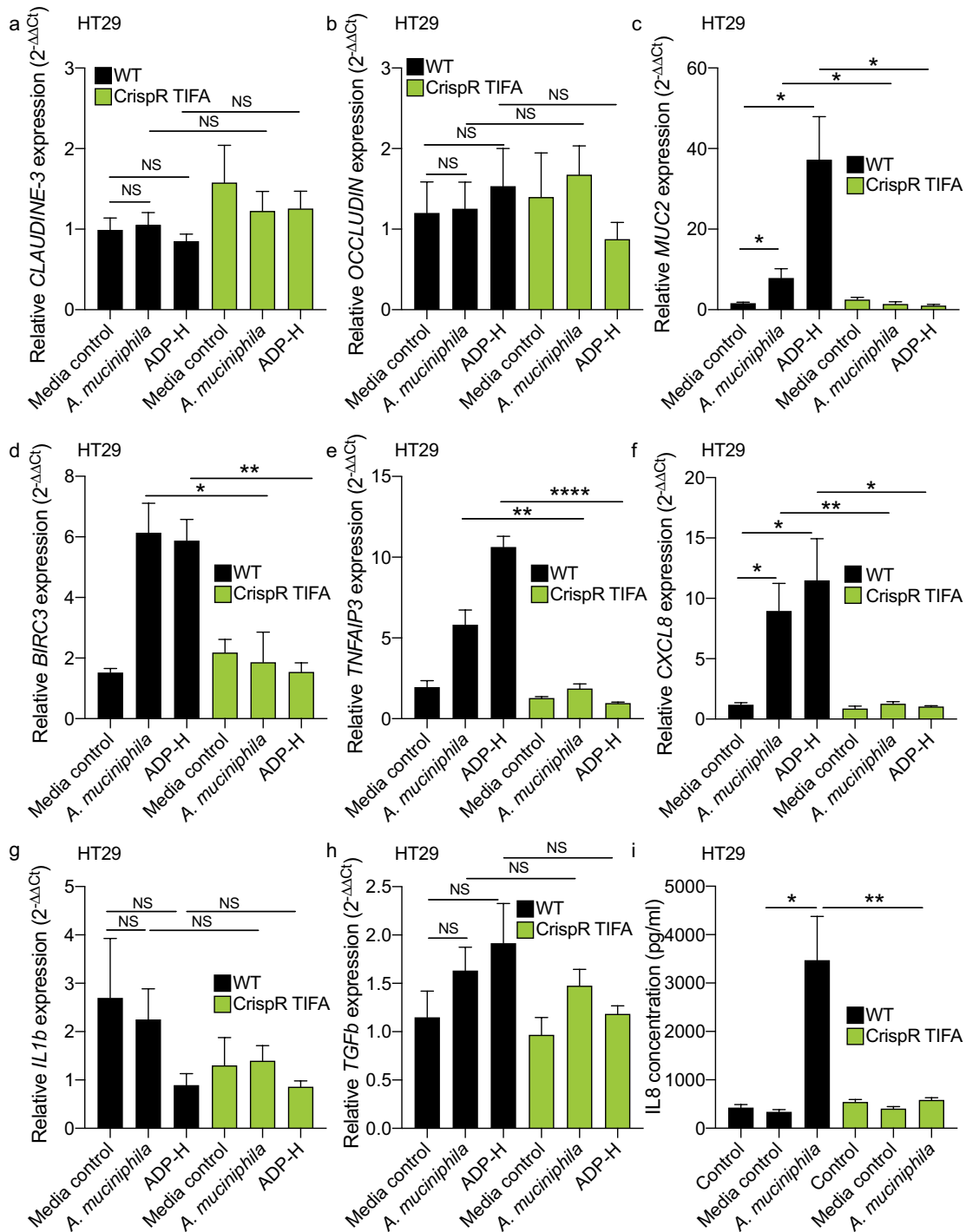
ADP-H improves the integrity of an enterocyte monolayer. In conclusion, our results strongly suggest that the activation of the *ALPK1/TIFA* pathway by ADP-H is involved in the beneficial impact of *A. muciniphila* on intestinal homeostasis.

## Discussion

A growing body of evidence suggests that commensal bacteria are not passive in the gastrointestinal tract but rather have an active impact on intestinal homeostasis. Indeed, balanced activation of PRRs by intestinal microbes plays a crucial role in maintaining a healthy epithelium by a wide range of functions, including survival, proliferation and differentiation of epithelial cells, production of mucus and antimicrobial peptides, repair of epithelial tissue at the sites of infection or damages, and maturation of immune cells. While numerous studies showed that gut bacteria control intestinal homeostasis through TLRs and NLRs,<sup>32,33</sup> the role of the newly discovered intracellular innate immune receptor *ALPK1* remains unknown. In this study, we report for the first time that the commensal bacterium, *A. muciniphila*, can activate the *ALPK1-TIFA-TRAF6* pathway in human intestinal cells by releasing extracellularly ADP-H related molecules. *A. muciniphila* is a Gram-negative commensal bacterium, with a relative abundance of 1–4% in the healthy human gastrointestinal tract that has

a positive impact on intestinal barrier integrity and global host health.<sup>7,8</sup> It has emerged as a potential beneficial bacterium positively impacting colitis, obesity and related metabolic disorders notably by improving intestinal barrier functions in mice models.<sup>6,7,10</sup> Although, the mechanisms of action of this bacterium are not yet fully elucidated, it has been shown that a pili-related protein Amuc\_1100 activates the innate immune receptor TLR2 and TLR4 and that this pathway could partially recapitulate the beneficial effect of *A. muciniphila*.<sup>29,34</sup> These studies illustrate that the role of TLRs is not limited to host defense against infections, and that activation of these innate immune receptors by a commensal bacterium is also implicated in the regulation of metabolic balance and gut barrier functions.

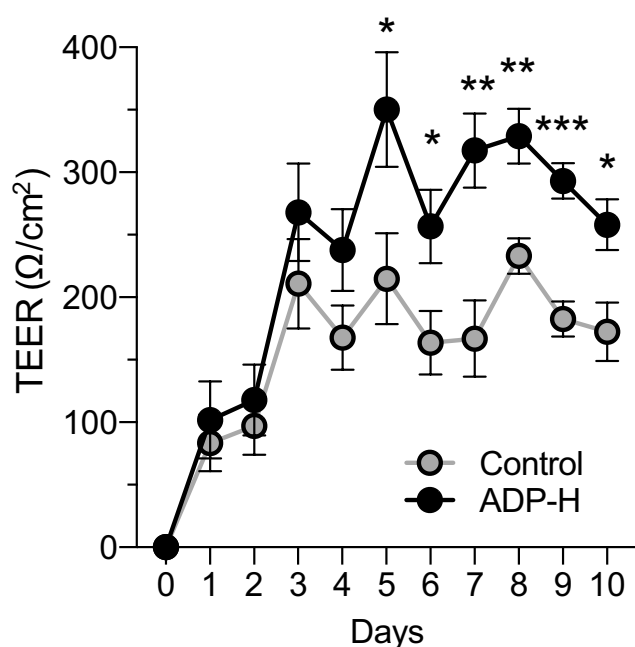
Activation of the *ALPK1/TIFA* axis has been described so far during infection by *S. flexneri*, *Y. pseudotuberculosis*, *H. Pylori*, *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *C. jejuni*, confirming the broad involvement of this pathway in innate immunity against Gram-negative bacteria.<sup>12–15,35,36</sup> Except for *N. meningitidis* and *C. jejuni*, it has been proposed that these bacteria activate the *ALPK1* by secreting ADP-H via the type III or a type IV secretion systems<sup>12,35</sup> or cytosolic bacterial replication.<sup>14</sup> We show, for the first time, that the commensal bacterium, *A. muciniphila* can release in the medium an heptose-related molecules able to activate the *ALPK1/TIFA* pathway, a feature that has only been



**Figure 6.** *A. muciniphila* activates the expression of *TNFAIP3*, *BIRC3*, *MUC2* and *CXCL8* via TIFA. A-H. Real-time PCR (RT-PCR) showing *CLAUDINE-3* (A), *OCCLUDIN* (B), *MUC2* (C), *BIRC3* (D), *TNFAIP3* (E), *CXCL8* (F), *IL1β* (G) and *TGFβ1* (H) relative expression to *GAPDH* in WT and *TIFA*<sup>-/-</sup> HT29, stimulated with ADP-H (10<sup>-6</sup>M), control media or *A. muciniphila* DSM22959 for 6 h. Results were normalized over *GAPDH* and expressed as 2<sup>-ΔCt</sup> toward unstimulated cells. I. IL-8 ELISA performed on WT or *TIFA*<sup>-/-</sup> HT29 cells treated with control media or *A. muciniphila* supernatant. IL-8 concentration was expressed in pg/ml. Data represent ≥3 independent experiments. Data analysis: unpaired t test was used, \*\*\*\*P < 0,0001; \*\*\*P < 0,001; \*\*P < 0,01; \*P < 0,05; P > 0,05 was considered as not significant (NS).

reported for *N. meningitidis* and *C. jejuni*.<sup>15,36</sup> The exact nature of the ALPK1-activating molecules released by *A. muciniphila* remains to be precisely characterized but our data strongly suggest that it is

ADP-H. First, extracellular ADP-H triggers ALPK1-mediated signaling in HEK293 cells whereas the related bacterial metabolite HBP needs to be electroporated.<sup>11,12,36</sup> Our results show that the



**Figure 7.** ADP-H improve the HT29 Cl.16E monolayer integrity. Transepithelial electrical resistance (TEER) of HT29 Cl.16E was measured daily and subtracted from the TEER measurement at day 0 before treatment with or without ADP-H ( $10^{-5}$ M). TEER was measured for 10 days and is expressed in Ohm/cm<sup>2</sup> as mean  $\pm$  SEM of biological quadruplets from 2 independent experiments. Data analysis: unpaired t test was used, \*\*\*\*P < 0,0001; \*\*\*P < 0,001; \*\*P < 0,01; \*P < 0,05; P > 0,05 was considered as not significant (NS).

ALPK1-activating molecule released by *A. muciniphila* does not require transfection, electroporation or artificial permeabilization to enter the cells, excluding HBP as a candidate. Second, ADP-H triggers rapid oligomerization of TIFA whereas HBP has to be first processed intracellularly by host adenylyltransferases in ADP-H-7-P to induce TIFAsome formation.<sup>11</sup> TIFAsome formation was monitored as soon as 30 min after incubation of permeabilized-cells with *A. muciniphila* supernatant, a feature of the ALPK1-ligand ADP-H.<sup>11</sup> Third, the bifunctional enzyme HldE, harboring heptokinase and ADP-transferase activities sequentially involved in ADP-H synthesis, is conserved in *A. muciniphila*. We showed that HldE from *A. muciniphila* restores ADP-H synthesis and ALPK1 activation when heterologously expressed in *E. coli*  $\Delta$ *hldE*. Last, ADP-H is sensitive to PDE from *C. adamanteus* and in line with this, we showed that the active compound present in the *A. muciniphila* supernatant was degraded by PDE, reinforcing the hypothesis that this bacterium releases ADP-H into its microenvironment.<sup>16</sup>

The mechanism by which ADP-H is released by bacteria remains to be elucidated.

Despite recent advances on the role of PRRs in human health, our understanding of how microbiota-derived PRR-ligands improve and restore intestinal homeostasis is still fragmentary. Our results show that *A. muciniphila*, via ADP-H-release and ALPK1 activation, induces the expression of *MUC2*, *BIRC3* and *TNFAIP3*, three genes involved in the maintenance of the intestinal barrier functions and of *Cxcl8*, a gene encoding for the pro-inflammatory cytokine IL8. *MUC2* is coding for mucin2, one of the main components of the mucus layer that tightly adheres to the epithelium and is involved in maintaining the integrity of intestinal barrier.<sup>37</sup> *BIRC3* and *TNFAIP3* are two anti-apoptotic genes involved in IEC proliferation, and crypt regeneration, and consequently, in epithelial renewal and barrier functions.<sup>38,39</sup> We showed that ADP-H improved integrity of the enterocyte monolayer, as shown by a significant increase of the TEER of the mucin-secreting clonal cell line HT29-Cl.16E upon addition of ADP-H. The increased in TEER could be explained by the upregulation of *MUC2* expression which could favor an increase of the mucin layer or that of the two anti-apoptotic genes which could promote cell proliferation or survival in this context. We analyzed *ALPK1* and *TIFA* expression in the different cell types of the intestine using published human single-cell transcriptomic data.<sup>26</sup> Interestingly, this analysis showed that these two genes are widely expressed in all intestinal epithelial cell types, including ISCs and differentiated cells, suggesting a role in intestinal development and IEC functions and development. We can therefore speculate on a potential impact of ALPK1 on intestinal homeostasis and hypothesize that the activation of this pathway by the gut microbiota may have a beneficial effect in pathological context where *A. muciniphila* has been reported as protective, such as obesity and colitis.<sup>7,10</sup> In support of this hypothesis, a recent study shows a role of ALPK1 as a regulator of intestinal inflammation in a model of pathobiont-induced colitis.<sup>40</sup>

As mentioned, *N. meningitidis* and *C. jejuni* are the only two pathogenic bacterial species that have been described as releasing heptose-related molecules in their microenvironment, albeit by unknown mechanisms. This feature, only observed for

pathogens so far, could be considered as a virulence factor since it activates the ALPK1-pathway and promotes an inflammatory response that is beneficial for some bacteria to complete infection. For instance, *C. jejuni* colonization of the intestinal tract is dependent on a severe intestinal inflammatory response.<sup>41</sup> Although *A. muciniphila*-secreted ADP-H induces the expression of the pro-inflammatory cytokine IL-8 *in vitro*, this process is likely not sufficient to induce detrimental intestinal inflammation *in vivo*.<sup>10</sup> Interestingly, IL-1 $\beta$  the other proinflammatory cytokine massively produced in bacterial infection, is not regulated by *A. muciniphila*. Potential consequences of IL-8 expression may be overbalanced by the upregulation of the anti-apoptotic genes BIRC3 and TNFAIP3 and by additional anti-inflammatory pathways triggered by the bacterium. For instance, *A. muciniphila* induces high amount of IL-10 in human derived PBMCs and increases the development of TEER *via* TLR2, suggesting that this bacterium has developed several mechanisms leading to anti-inflammatory and protective properties.<sup>29</sup> In support of this, a study showed that *A. muciniphila* colonized-mice did not develop any signs of inflammation and that this bacterium protects in DSS-induced colitis.<sup>10</sup> Several non-exclusive parameters may explain the differential inflammatory responses observed *in vivo* between *A. muciniphila* and the ADP-H-secreting pathogen *C. jejuni*. They include i) the quantities of ADP-H produced, ii) the different bacterial niches colonized by these bacteria in the intestine, iii) the production of additional molecules or virulence factors that may antagonize or synergize with ALPK1/TIFA axis.

NF- $\kappa$ B signaling can be divided into two pathways: the classical and the alternative pathways. NF- $\kappa$ B activation impacts multiple cellular processes including inflammatory, proliferative and both pro- or anti-apoptotic responses. Despite these effects that may appear contradictory, experiments involving targeted deletion of genes of both NF- $\kappa$ B pathways demonstrated that activation of NF- $\kappa$ B in IECs is key for limiting intestinal inflammation in colitis-induced animal models.<sup>42,43</sup> The mechanisms of the conventional NF- $\kappa$ B pathway involved in intestinal homeostasis has been extensively studied and was shown to be associated with the activation of antimicrobial peptides, the induction of the expression of anti-apoptotic genes and the maintenance of Paneth cell

numbers.<sup>42</sup> The role of the alternative NF- $\kappa$ B pathway in intestinal homeostasis is less documented. Mouse models suggest that the alternative NF- $\kappa$ B pathway components, NIK and I $\kappa$ ka, are required for the development of Peyer's patches and secondary lymphoid structure, maintenance of M-cells and establishment of a protective IgA response.<sup>44</sup> Interestingly, a recent study demonstrated that ADP-H-dependent activation of the ALPK1/TIFA pathways results in the activation of both conventional and alternative NF- $\kappa$ B signaling in a TRAF6 and TRAF2 dependent manner, respectively.<sup>45</sup> Our work mainly focused on the activation of the conventional pathway by *A. muciniphila* but we cannot exclude that the alternative pathway is also activated. In addition to ADP-H, *A. muciniphila* produces diverse molecules that activate PPRs.<sup>29</sup> Therefore, the overall effect of *A. muciniphila* on intestinal epithelium results from a combination of synergistic or antagonistic interactions between these metabolites, and the properties of these molecules taken individually may not reflect their collective effect on intestinal homeostasis *in vivo*. How precisely is ALPK1 involved in the beneficial impact of *A. muciniphila* on intestinal homeostasis? Additional work using ALPK1-targeted deletion in animal models is needed to properly answer this question. At this point, our results show that the beneficial commensal bacterium *A. muciniphila* activates this newly emerging pathway of innate immunity and provide the first evidence to position the ADP-H sensor ALPK1 as a potential new hub for the host-microbiota dialogue.

## Materials and methods:

### Cell Culture and reporter systems

HT29 (American Type culture Collection, ATCC) and HEK293 (Invivogen) cells were grown in RPMI 1640 GlutaMAX™; HeLa cells (ATCC) and HT29-Cl.16E (kind gift of Anne Jarry, CRCINA)<sup>30</sup> in DMEM GlutaMAX™ medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Eurobio), with 50 IU/mL penicillin, 50  $\mu$ g/mL streptomycin and 10%, 100 mM HEPES, 10 mM non-essential amino acids. Cells were grown at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. All culture media and supplements were supplied by Gibco (ThermoFisher). Mycoplasma contamination was regularly tested using MycoAlert (Lonza) and

PlasmoTest (Invivogen). HT29 and HEK293 stably expressing secreted alkaline phosphatase (SEAP, pNifly, Invivogen) reporter gene were used for NF- $\kappa$ B activation monitoring (HT29-NF- $\kappa$ B and HEK-NF- $\kappa$ B respectively)<sup>17</sup> and Invivogen). HeLa cells stably expressing TIFA-GFP were used to monitor TIFA oligomerization<sup>11</sup>.

### **Reagents and cytokines**

All agonists, drugs and inhibitors were dissolved in DMSO or water following the manufacturer's recommendations. Digitonin was used at a concentration of 2,5  $\mu$ g/ml for 30 min (Interchim). Dynasore was used at a concentration of 80  $\mu$ M for 1h (Sigma-Aldrich). NOD1 inhibitor (ML130, Sigma) and NOD1 ligand (IE-DAP, Invivogen) were used at 10 $\mu$ M and 10 $\mu$ g/ml respectively. ADP-heptose (ADP-H, 9020852, J&K Scientific and Invivogen) was used at concentration ranging from 10<sup>-8</sup> to 10<sup>-5</sup>M. HBP was synthesized as previously described and was used at a concentration 10<sup>-3</sup>M<sup>11</sup>.

### **Culture of commensal bacteria and preparation of supernatants**

Bacterial strains including Akkermansia muciniphila strains (DSM22959 from human origin and DSM26127 from mice origin) are from DSMZ-German Collection or from the collection de l'Institut Pasteur (CIP). Bacteria were grown in specific media and anaerobic culture conditions accordingly to the Hungate method<sup>47</sup> as mentioned in the supplementary Table S1. Bacterial cultures were cultured 24h to reach the maximum optical densities (OD600, see Table S1). Bacterial supernatants were harvest after centrifugation at 5,000  $\times$  g for 10 min and filtered on a 0.22  $\mu$ m PES filters and stored at -80°C. Quality controls were performed using Gram staining method, aerobic growth test and fresh observation on microscope. Non-inoculated culture medium served as control.

### **Cell stimulation and reporter system assays.**

For each experiment, HEK-NF- $\kappa$ B (WT, ALPK1<sup>-/-</sup>, TIFA<sup>-/-</sup>, TRAF6<sup>-/-</sup>, MYD88<sup>-/-</sup>) and HT29-NF- $\kappa$ B (WT and TIFA<sup>-/-</sup>) cells were seeded at 3.10<sup>4</sup> cells

per well in 96-well plates 24 h prior to incubation with bacterial supernatants or reagents. The cells were stimulated for 24 h with bacterial supernatants or controls (RPMI and non-inoculated bacteria culture medium) in a total culture volume of 100  $\mu$ L per well prior to the reporter assay. When indicated cells were incubated with digitonin (2,5  $\mu$ g/ml for 30 min), dynasore (80 $\mu$ M for 1 h), ML130 (10  $\mu$ M), IE-DAP (10  $\mu$ g/ml) or ADP-H (10<sup>-8</sup> to 10<sup>-6</sup> M). Secreted embryonic alkaline phosphatase (SEAP) was revealed with the Quanti-Blue reagent (Invivogen) using microplate reader (655 nm Infinite 200, Tecan). The NF- $\kappa$ B activation was normalized to the controls, i.e., the unstimulated cells. Experiments were performed in triplicates for at least three independent assays. Cell viability was monitored by MTS measurement using the CellTiter 96 Aqueous One solution (Promega) according to the manufacturer's recommendations.

### **CRISPR-Cas 9 deletion of ALPK1, TIFA, MYD88 and TRAF6 in HEK293 and HT29 cells.**

We used the plentiCRISPR version 2 (Addgene plasmid 52961;<sup>48</sup> and the target site GGACCAGCGCTGCAGAGGTG to obtain the plentiCRISPRv2-ALPK1 for ALPK1 deletion as published by et Zimmerman et al.<sup>35</sup>. Oligos containing ALPK1 sgRNA were cloned into lentiCRISPRv2 (Addgene #52961) using oligos previously published: CACCGGGACCAGCGCTGCAGAGGTG (ALPK1 up-Oligo), AAACCACCTC TGCAGCGCTGGTCCC (ALPK1 down-Oligo)<sup>35</sup>. 7.10<sup>5</sup> HEK293T cells were seeded with DMEM without antibiotics in 6cm<sup>2</sup> plate. The next day, cells were transfected with a mixture of 750ng psPAX2 (Addgene #12260), 250ng of pMD2.G (Addgene #12259) and 1 $\mu$ g of pLentiCRISPRv2-ALPK1 using Lipofectamine 2000 (Invivogen) diluted in OptiMEM (Gibco). Medium was changed after 18 h of transfection and lentiviral particles were harvested 2-3 days after transfection and filter 0.45 $\mu$ M before storage at -80°C. HEK-NF- $\kappa$ B reporter cells were infected with lentiviral particles in presence of 8  $\mu$ g/ml polybrene (Millipore). Fresh medium was added 6h later and selection with puromycin 1  $\mu$ g/ml started 24 h later for one week. Clonal cells were tested for NF- $\kappa$ B activity

upon stimulation with 1Kd-filtered *E. coli* lysate before genotyping of genomic DNA by PCR using oligos previously published: GTGGTAGCTGTGC TACTGCAAG (fw) and ACTCATCTCTCGGTGG TCATCT (rv)<sup>35</sup>. HEK-NF- $\kappa$ B clone with the exact same insertion as published was selected<sup>35</sup>.

The TIFA, TRAF6 and MYD88 genes were disrupted using All-in-one sgRNA clone co-transfection (DNA-In, MTI-GlobalStem) of a plasmid containing Cas9 nuclease and specific template sgRNAs (pCRISPR-CG01) and a second plasmid containing homology domains for the targeted gene and a GFP and Puromycine cassette (pDonor-D01) in NF- $\kappa$ B-HEK and NF- $\kappa$ B-HT29 cell lines (GeneCopoeia). The target sites were AATGCGACC CGACCGCGCTG for MYD88 gene, CAGGATGGT AAACCGTCATC for TIFA gene, ATCTTTTGTTA CAGCGCTAC for TRAF6 gene. Transfected cells were selected with puromycin and clonally selected for the cassette insertion by cytometry (GFP positive cells) and PCR. The PCR oligos used were: Myd88-for: CGCTTCTCGGAAAGCGAA, Myd88 rev: GTAGAAAGAAGGGCTGCAT, Tifa for: AATCA AAGTTCTCAATGCCTC, Tifa rev: TTGTGAACT GATGTGACCAAA, Traf6-for: GTGTGTTACTTA TAAAGGCATT, Traf6-rev: AAGGGACTGGATTT TGGATA, Insert-for: GCTATACGAAGTTATAA TCTAGA.

### **Treatments of *A. muciniphila* supernatant.**

*A. muciniphila* supernatants were treated with alkaline phosphatase (CIP, 100u/ml) or phosphodiesterase (PDE, 30 mU/mL) from *Crotalus adamanteus* for 30 min, at 37°C as published followed by a 3kDa-sieved filter step to eliminate the enzymes<sup>16</sup>. Control media were treated similarly. Cells were stimulated with the treated supernatants and the NF- $\kappa$ B signal was monitored.

### **Cloning and expression of *A. muciniphila* HldE into *E. coli* $\Delta$ HldE.**

*E. coli* K-12 WT and  $\Delta$ HldE *E. coli* (JW3024) are from the Keio collection<sup>49</sup> (Dharmacon). hldE from *A. muciniphila* was amplified using the following primers: (i) AhldEfor\_NheI (5'-GGGGGCTAGC AGGAGGTAAATAATGAACCGGCTGCATACA-T-3') creating a new NheI restriction site (GCTAGC)

and adding a Shine-Dalgarno sequence (AGGAGG) located 6 bases upstream of the start codon ATG and (ii) AhldErev\_HindIII (5'-GGGGAAGCTTTCAT TCCGGGCTGCTTTTC-3') creating a new HindIII restriction site (AAGCTT). The 1523 pb fragment was amplified using Phusion High-Fidelity DNA polymerase, A-tail with GoTaq polymerase and cloned in pGEM<sup>®</sup>-T Easy vector (Promega Corporation) to generate pGEM-T-AhldE. After NheI HindIII restriction of the pGEM-T-AhldE, the AhldE fragment was cloned into NheI-HindIII-restricted pBAD24 generated pBAD24-AhldE vector (amp). pBAD24-AhldE and the empty vector pBAD24 were purified and used to electroporate *E. coli*  $\Delta$ HldE. Overnight bacterial cultured were washed in PBS and resuspended at an OD = 1, boiled for 30 min and stored at -20°C until use.

### **Real-Time PCR**

HT29 or HEK cells were seeded in 6 well culture plates at densities of  $1.10^6$  per well 24h before stimulation and total RNA was extracted using RNeasy mini-Kit (Qiagen) according to the manufacturer's recommendations with Dnase I treatment (R&D). cDNA was synthesized from 2 $\mu$ g of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 100ng were used to conduct qPCRs on ABI Prism 7700 (Applied Biosystems) or StepOnePlus Real-Time PCR System (ThermoFischer Scientific). The following Taqman Gene expression assay probes were used: GAPDH Hs02758991\_g1, ALPK1 Hs01567926, TIFA Hs00385268, MUC2 Hs03005103, CLAUDINE-3 Hs00265816, OCCLUDIN Hs05465837, BIRC3 Hs00985029, TNFAIP3 Hs00234713, CXCL8 Hs00174103\_m1, IL1 $\beta$  Hs01555410\_m1, TGF $\beta$ 1 Hs00998133\_m1. GAPDH was used for normalization. comparisons were done with  $\Delta\Delta$ Ct. Samples were tested in experimental duplicates and at least biological triplicates.

### **siRNA assay**

HeLa cells seeded in 96-well plates (8000 cells/well) were transfected with 20 nM siRNA as previously described<sup>13</sup>. Cells were transfected with a validated ALPK1 siRNA (s37074, Ambion, validation data available on Ambion's website) and a non-targeting



sequence (4390843, Ambion). For the ALPK1 rescue experiment, 48 h after siRNA transfection, cells were transfected with an siRNA-resistant ALPK1 cDNA construct (pCMV-ALPK1) or an empty vector (pCMV) using FuGENE 6 (Roche) prior TIFAsome analysis as previously described<sup>11</sup>.

### **TIFAsome assay and quantification**

HeLa TIFA-GFP cells were seeded in 96-well plates at least one day before experiment. Cells were washed in permeabilization buffer containing 100 mM KCl, 3 mM MgCl<sub>2</sub>, 50 mM Hepes, 0,1 mM DTT, 85 mM sucrose and 0,2 BSA and 0,1 mM ATP. Cells were then incubated with digitonin alone as control or digitonin plus HBP, ADP-H, medium control or bacterial supernatant for 30 min in permeabilization buffer. To monitor TIFA oligomerization, cells were fixed at 30 min with 4% PFA or washed and incubated for 5,5 h in DMEM complete medium before fixing. Images were acquired with an ImageXpress Micro (Molecular devices). Image analysis and TIFAsomes quantification were performed using the custom module editor of MetaXpress as previously published<sup>11</sup>.

### **ELISA**

HT29 cells were seeded at  $1 \times 10^6$  cells per well in 6 well-plates 24 h prior incubation with bacterial supernatants, non-inoculated bacterial media or ADP-H. Supernatants were collected 24 h later and immediately assessed by ELISA following manufacturer instructions using the IL8 ELISA kit (Biolegend).

### **Western Blot**

HT29 or HEK cells were washed twice and lysed in buffer (1% NP40, 150mM NaCl, 50mM Tris-HCL pH8, 5mM EDTA, 1 x Complete Protease Inhibitor Cocktail (Roche). Nucleus were eliminated by centrifugation for 10 minutes 4°C at 17500g. Protein extracts were run in SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked overnight in TBS 0.1% tween 4% skim milk or BSA (Sigma). Primary antibodies were incubated overnight at 4°C, **MYD88 (Santa Cruz)**, **TRAF6 (Biolegend)**,

**ALPK1, Actin (1:2000, AC-40 Sigma)**. Secondary (Goat anti-Rabbit IgG HRP (P0448) and Goat anti-mouse HRP (P0447) from Dako) antibodies were successively added for 1h before detection with the Clarity Western ECL Substrate using the Chemidoc MP System (Bio-Rad).

### **scRNAseq analysis.**

Single-cell transcriptomics data from intestinal epithelial cells were obtained from gutcellatlas.org/#datasets<sup>46</sup> and normalised using Seurat (version 4.0.6<sup>50</sup>). Only cells annotated as adult epithelial cells were kept for further analysis and cell subtypes were grouped to correspond to the nine main intestinal epithelial cell types. Individual normalized cell expression and average expression across cell types were calculated and plotted using R 4.1.

### **Transepithelial Electrical Resistance (TEER) measurement.**

HT29-Cl.16E cells were cultured at confluency to differentiate them into mucus secreting cells<sup>30</sup>. HT29-Cl.16E cells were seeded at density of  $1 \times 10^6$  cells/well on inserts in the HTS Transwell 24-well permeable support system (0,4 µm pore polyester membrane, Corning) and placed into 24-wll tissue culture plates. After one day, ADP-H ( $10^{-5}$  M) was added to the wells (Day 1) and then every two days when the medium was changed. The TEER was measured daily with the Millicell ERS-2 Voltohmmeter system (Millipore) and the STX100 Electrodes (Corning) and expressed in Ohm/cm<sup>2</sup>.

### **Statistical Analysis**

All experiments were conducted independently at least three times, otherwise specified, in triplicate and the results are expressed as mean  $\pm$  SEM fold change towards control condition. Data were represented and analyzed using GraphPad Prism (GraphPad software). The differences between individual groups were verified using an unpaired t test or one-way ANOVA followed by Tukey's multiple comparisons test. P-values are indicated as follows: \*\*\*\*P<0,0001; \*\*\*P<0,001; \*\*P<0,01; \*P<0,05; P>0,05 was considered as not significant (ns).

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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Conceived and designed the experiments: CMG, NL, DGW, CA; intellectual contribution: SGO, JD, HMB, CA; data acquisition: CMG, DGW, AL, LM, VT, AR, FBC, VB, TQ, AJ, CA, NL; data analysis, statistical analysis and interpretation: CMG, DGW, PL, CA, NL; wrote the paper: CMG, CA, NL; edited and revised the manuscript: DGW, SGO, JD, HMB.

## Data availability statement

All data are contained in the article and the supporting information. Single-cell transcriptomics data from intestinal epithelial cells were obtained from gutcellatlas.org/#datasets from Elmentaite *et al.* 2020.<sup>46,47,48,49,50</sup>

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