Gene-Microbiota Interactions Contribute to the Pathogenesis of Inflammatory Bowel Disease

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Abstract

Inflammatory bowel disease (IBD) is associated with risk variants in the human genome and dysbiosis of the gut microbiome, though unifying principles for these findings remain largely undescribed. The human commensal Bacteroides fragilis delivers immunomodulatory molecules to immune cells via secretion of outer membrane vesicles (OMVs). We reveal that OMVs require IBD-associated genes, ATG16L1 and NOD2, to activate a non-canonical autophagy pathway during protection from colitis. ATG16L1-deficient dendritic cells do not induce regulatory T cells (T_{reg}) to suppress mucosal inflammation. Immune cells from human subjects with a major risk variant in ATG16L1 are defective in T_{reg} responses to OMVs. We propose that polymorphisms in susceptibility genes promote disease through defects in ‘sensing’ protective signals from the microbiome, defining a potentially critical gene-environment etiology for IBD.

Intestinal microbiota modulate development and function of the immune system, and play a critical role in inflammatory bowel disease (IBD), a family of idiopathic intestinal disorders including Crohn’s disease (CD) and ulcerative colitis (UC) (1–6). Concordance rates of 40-50% between monozygotic twins implicate gene-environment interactions contribute to CD (7-10), albeit in ways that are poorly understood. Advances in DNA sequencing

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technologies have empowered unprecedented insights into the human genome and the gut microbiome in IBD, enabling detailed genomic characterization of patients (11) and chronicling alterations in the composition and gene content of the gut microbiome (dysbiosis) (12).

Close to 200 risk loci have been proposed for CD, with several susceptibility genes linked to the regulation of autophagy (e.g., \textit{ATG16L1}) (13-15) or to microbial sensors that activate autophagy (e.g., \textit{NOD2}) (16-18). While previous studies have shown that disruption of \textit{ATG16L1} and \textit{NOD2} impacts CD susceptibility through defects in microbial clearance (19-23), recent reports reveal that immune cells impaired in autophagy are hyper-inflammatory (24-29). This suggests that deficiencies in \textit{ATG16L1} or \textit{NOD2} may contribute to CD risk through impaired anti-inflammatory responses, a hypothesis not mutually exclusive with microbial clearance functions.

The microbiome of CD patients is altered, with emerging evidence for cause and effects relationships to disease. Among other recent examples of host-microbe interactions (3, 5, 6), the human commensal \textit{Bacteroides fragilis} has evolved beneficial immunomodulatory properties. During colonization of mice, \textit{B. fragilis} capsular Polysaccharide A (PSA) is packaged in outer membrane vesicles (OMVs) and delivered to intestinal dendritic cells to induce interleukin-10 (IL-10) production from CD4$^+$Foxp3$^+$ regulatory T cells (T\textsubscript{regs}), which protect from experimental colitis (30-32). To explore gene-environment interactions during host-microbiota symbiosis, we tested if genetic pathways linked to CD are involved in the immune response to \textit{B. fragilis} OMVs.

Bone marrow-derived DCs (BMDCs) differentiated from wild-type (WT) and \textit{ATG16L1}-deficient (\textit{Atg16l1}\textsuperscript{fl/fl} \textit{Cd11cCre}; \textit{Atg16L1}\textsuperscript{ΔCD11c}) mice were pulsed with OMVs harvested from wild-type \textit{B. fragilis} (WT-OMV) or an isogenic mutant lacking PSA (ΔPSA-OMV), and co-cultured with CD4$^+$ T cells. As previously reported (33), WT-OMVs, but not vehicle or ΔPSA-OMVs, promote IL-10 production (Fig. 1, A to C, figs. S1 and S2). Conversely, \textit{ATG16L1}-deficient DCs do not support IL-10 production in response to WT-OMVs (Fig. 1, A to C). We observe similar results using \textit{Atg16l1}\textsuperscript{fl/fl} \textit{LysMCre} mice (fig. S3). Purified PSA does not require \textit{ATG16L1} for its activity (Fig. 1, A and C, fig. S2). Next, we tested functional outcomes using \textit{in vitro} T cell suppression assays. T\textsubscript{regs} isolated from co-cultures with \textit{Atg16L1}\textsuperscript{ΔCD11c} BMDCs treated with \textit{B. fragilis} OMVs exhibit impaired suppressive activity (Fig. 1D and fig. S2A). Neither WT-OMVs nor pure PSA have any effect on IL-10 production among CD4$^+$Foxp3$^-$ type 1 regulatory T cells (fig. S4). \textit{ATG16L1}, \textit{ATG5} and \textit{ATG7} are components of the autophagy elongation complex; BMDCs deleted in these genes likewise do not induce IL-10 production from T\textsubscript{regs} (fig. S5). Further, recent reports reveal a role for autophagy components in T\textsubscript{reg} homeostasis (34, 35). Our findings indicate that \textit{ATG16L1}-deficient DCs fail to respond to \textit{B. fragilis} OMVs, demonstrating that autophagy components in DCs are required for commensal-driven T\textsubscript{reg} induction and function.

\textit{ATG16L1}, \textit{ATG5} and \textit{ATG7} participate in both canonical and non-canonical autophagy pathways (36). Interestingly, the classical autophagy-specific genes \textit{Ulk1}, \textit{Fip200} or \textit{Atg14} are not required for CD4$^+$Foxp3$^+$IL-10$^+$ T\textsubscript{reg} induction upon WT-OMV treatment (fig. S6). We hypothesized that OMVs utilize the non-canonical autophagy pathway, LC3-associated
phagocytosis (LAP), which is specifically activated by microbial ligands delivered as particles rather than soluble molecules. LAP activation requires RUBICON, which represses canonical autophagy (36). Rubicon+/− but not Rubicon−/− BMDCs display increased accumulation of lipidated, membrane-bound LC3-GFP (LC3-II) upon B. fragilis WT-OMV treatment (Fig. 1E). As expected, neither ΔPSA-OMVs nor purified PSA are able to activate LAP (fig. S7). Moreover, treatment of Rubicon−/− DCs fails to induce T_{reg} responses (Fig. 1F). As RUBICON is upstream of ATG16L1 signaling, OMVs preferentially utilize the non-canonical autophagy pathway LAP to mediate tolerogenic responses to B. fragilis. Further, these data suggest a reconsideration of previous literature assigning the role of ATG16L1 in IBD to defects exclusively in autophagy.

As a CD-risk gene, we investigated the in vivo requirement for ATG16L1 in CD11c+ DCs during OMV-mediated protection from experimental colitis. Indeed, WT mice treated by oral gavage with WT-OMVs are protected from 2,4-dinitrobenzenesulfonic acid (DNBS) colitis (33), whereas Atg16L1ΔCD11c mice exhibit acute weight loss and increased mortality similar to untreated mice (Fig. 2A and fig. S8A). WT, but not Atg16L1ΔCD11c mice, orally administered OMVs are protected from shortening of the colon, a hallmark of colitis models (Fig 2B), with colitis scoring and cytokine profiles verifying protection from disease (Fig. 2C and fig. S8B). Prevention of colitis is not due to an overall defect in T_{reg} development in Atg16L1ΔCD11c mice (fig. S9). Further, while proportions of CD4+Foxp3+ cells are comparable in all groups of mice during colitis (fig. S10), Atg16L1ΔCD11c mice produce significantly less IL-10 from gut Foxp3+ T_{regs} compared to WT mice following WT-OMV treatment (Fig. 2D and fig. S8C). Thus, WT-OMVs require ATG16L1 within DCs to induce IL-10 expression from Foxp3+ T_{regs} and to suppress intestinal inflammation in a colitis model.

In addition to impaired IL-10 production in response to OMV treatment, Atg16L1ΔCD11c mice display an increase in IL-17A expression (Fig. 2E), but not IFN-γ (fig. S11), among mucosal CD4+Foxp3+ T cells during colitis. Further, in vitro co-cultures of OMV-pulsed Atg16L1ΔCD11c BMDCs result in impaired IL-10 expression among T_{regs} (Fig. 1C), and increased IL-17A production in CD4+Foxp3+ T cells (fig. S12). Interestingly, while OMVs from other enteric bacteria each elicited a unique ATG16L1-dependent immune profile, only B. fragilis OMVs exclusively induce an anti-inflammatory response (fig. S13). Together, these data suggest ATG16L1-deficiency in DCs alters the quality of the T cell response to OMVs.

As DCs coordinate adaptive immunity, we sought to determine how Atg16L1ΔCD11c DCs are impaired in promoting tolerogenic responses. Following OMV stimulation, we observe no differences by WT or Atg16L1ΔCD11c DCs in internalizing OMVs, or in surface expression of MHC II, CD80 and CD86 (fig. S14) (27). However, stimulation with OMVs results in an increase transcription of multiple pro-inflammatory cytokines in Atg16L1ΔCD11c DCs compared to WT cells (fig. S15). These data are consistent with previous reports of a hyper-inflammatory response in ATG16L1-deficient macrophages and DCs stimulated with other microbial ligands (24, 26). Abrogation of T_{reg} responses by ATG16L1-deficient DCs is likely due to increased pro-inflammatory cytokine production, which may impair DC-T cell interactions. Atg16L1ΔCD11c mice do not display more severe...
colitis than WT mice in the absence of OMV treatment (Fig. 2), suggesting that lack of protection is not due to more fulminant inflammation, but rather an inability to induce T\(_{\text{regs}}\) in mice deficient in ATG16L1 among CD11c\(^+\) DCs.

\(NOD2\) encodes for an intracellular sensor of bacterial peptidoglycan, and polymorphisms in this gene contribute to the largest fraction of genetic risk for CD (13). \(NOD2\) has been shown to physically recruit ATG16L1 (20, 21), a process that is impaired in human cells homozygous for a \(NOD2\) frameshift mutation (20). Accordingly, \(Nod2^{-/-}\) BMDCs pulsed with WT-OMVs are unable to support IL-10 production from Foxp3\(^+\) T\(_{\text{regs}}\) during \textit{in vitro} co-cultures (Fig. 3, A and B), revealing a crucial role for \(NOD2\) signaling in microbiome-mediated immune tolerance. This notion is supported with \textit{in vivo} studies showing that \(Nod2^{-/-}\) mice are not protected from colitis by WT-OMV treatment (Fig. 3, C and D).

Similar to \(Atg16L1^{\Delta CD11c}\) animals, \(Nod2^{-/-}\) mice produce significantly less IL-10 from Foxp3\(^+\) T\(_{\text{regs}}\) of the MLN following WT-OMV treatment (fig. S16A), while proportions of T\(_{\text{regs}}\) remain unchanged during DNBS colitis (fig. S16B). Previous studies have shown that Toll-like receptor 2 (TLR2) is required for the PSA response (33, 37). While the role of \(NOD2\) in inducing LAP is currently unknown, signaling through TLR2 potently activates LAP (36, 38). \(B.\ fragilis\) OMVs induce reactive oxygen species (ROS) from WT DCs, a known product of LAP activation (36), but at significantly reduced levels in \(Nod2^{-/-}\) or \(Tlr2^{-/-}\) DCs (fig. S17). Though further studies are needed to define the mechanism of LAP activation by OMVs, these data reveal that \(NOD2\) and \(ATG16L1\) may cooperate as part of a common pathway to promote anti-inflammatory immune responses to the microbiome.

To extend and validate gene deletion approaches, we tested responses to OMVs by immune cells carrying the CD-associated variant of \(ATG16L1\) (13, 14, 39). The \(ATG16L1\) T300A variant leads to protein instability and altered cellular responses (23). BMDCs from transgenic mice expressing the T300A allele are also unable to promote IL-10 expression from Foxp3\(^+\) T\(_{\text{regs}}\) in response to WT-OMVs (fig. S18A). Further, \(ATG16L1\) T300A transgenic mice are not protected from DNBS colitis and do not mount a potent T\(_{\text{reg}}\) response when administered WT-OMV compared to WT mice (fig. S18, B to F). These findings prompted us to investigate if human immune cells from CD patients with the \(ATG16L1\) T300A risk variant (table S1) are also defective in promoting Foxp3\(^+\) T\(_{\text{reg}}\) development by \(B.\ fragilis\) OMVs. Monocyte-derived dendritic cells (MoDC) from CD patients and healthy controls harboring either the protective allele (T300) or the risk allele (T300A) were pulsed with OMVs or PSA and co-cultured with syngeneic CD4\(^+\) T cells. Consistent with our mouse data, human cells homozygous for the risk allele are unable to support induction of IL-10 from Foxp3\(^+\) T\(_{\text{regs}}\) by WT-OMVs compared to MoDCs carrying the protective allele (Fig. 4). Remarkably, all samples tested display the predicted outcome based on genotype, and not disease status. However, cells from most subjects, regardless of genotype, respond to purified PSA (Fig. 4). Collectively, we conclude that mouse and human DCs require functional ATG16L1 for induction of CD4\(^+\)Foxp3\(^+\)IL-10\(^+\) T\(_{\text{regs}}\) in response to \(B.\ fragilis\) OMVs.

IBD impacts over 1.5 million people in the US, with rates of diagnosis increasing and treatment options remaining limited (40, 41). The etiology of IBD is complex and incompletely resolved (1). We describe herein that interactions between genetic (\(ATG16L1/\)
and environmental (microbiome) factors cooperate to promote beneficial immune responses. *B. fragilis* OMVs utilize LAP, an ATG16L1-dependent cellular trafficking and signaling pathway, to induce mucosal tolerance. The hyper-inflammatory responses that occur with mutations in ATG16L1 likely alter antigen-processing pathways and impair signaling by DCs to T cells, and may explain why CD-associated polymorphisms abrogate $T_{reg}$ induction by OMVs. Collectively, discovery of genetic circuits co-opted by the microbiome to engender health provides unprecedented functional insights into gene-environment interaction relevant to the pathogenesis of IBD. We propose an additional role for genes previously implicated in killing bacteria—namely, mutations in genetic pathways linked to IBD result in an inability to sense and/or respond to beneficial microbes. This hypothesis may represent a new perspective for the etiology of microbiome-related diseases.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References and Notes**


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Fig. 1. ATG16L1 signals via a non-canonical autophagy pathway during OMV-mediated T<sub>reg</sub> induction

(A) ELISA for IL-10 production during DC–T cell co-cultures with WT or Atg16L1<sup>ΔCD11c</sup> BMDCs treated with PBS, <i>B. fragilis</i> WT-OMV, ΔPSA-OMV or purified PSA. (B and C) Representative flow cytometry plots (B) and frequency (C) of CD4<sup>+</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> T<sub>regs</sub> from DC–T cell co-cultures with WT or Atg16L1<sup>ΔCD11c</sup> DCs treated with PBS, <i>B. fragilis</i> WT-OMV, ΔPSA-OMV or purified PSA. (D) T cell suppression assay analyzing in vitro generated T<sub>regs</sub> from WT or Atg16L1<sup>ΔCD11c</sup> DCs treated with WT-OMVs. (E) Quantification of LC3-GFP accumulation by <i>B. fragilis</i> WT-OMV treatment of Rubicon<sup>+/−</sup> or Rubicon<sup>−/−</sup> DCs. Representative flow cytometry histogram plot (inset). PBS, grey; WT-OMV, blue. (F) Frequency of CD4<sup>+</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> T<sub>regs</sub> from Rubicon<sup>+/−</sup> or Rubicon<sup>−/−</sup> DC–T cell co-cultures treated with PBS, <i>B. fragilis</i> WT-OMV, ΔPSA-OMV or purified PSA. Error bars represent S.E.M. * <i>p</i> < 0.05, *** <i>p</i> < 0.001, **** <i>p</i> < 0.0001. Two-way ANOVA, followed by Tukey’s post-hoc analysis. Data are representative of at least 2 independent experiments.
Fig. 2. *B. fragilis* OMVs require ATG16L1 in CD11c+ DCs for protection from colitis

(A and B) Weight loss (A), colon length and gross pathology (B) of WT and Atg16L1ΔCD11c mice orally treated with PBS or *B. fragilis* WT-OMV during DNBS colitis. Sham groups were treated with ethanol. (C) Colitis scores by a blinded pathologist using a standard scoring system, and representative H & E images. Scale bar represents 100 μm. (D and E) Mesenteric lymph node (MLN) lymphocytes isolated post-DNBS analyzed for IL-10 (D) and IL-17A (E) production among CD4+Foxp3+ T_{reg}s, as assessed by flow cytometry. Error bars represent S.E.M. * p < 0.05, *** p < 0.001, **** p < 0.0001. Two-way ANOVA, followed by Tukey’s post-hoc analysis. Data are representative of at least 3 independent experiments, with 3-9 mice/group.
Fig. 3. NOD2 is required for OMV-mediated Tregs induction and protection from colitis

(A and B) Representative flow cytometry plots (A) from WT-OMV (left) and ΔPSA-OMV (right) treated BMDCs co-cultured with CD4+ T cells, and frequency (B) of CD4+Foxp3+IL-10+ Tregs from DC–T cell co-cultures. (C and D) Weight loss (C), colon length and gross pathology (D) of WT or Nod2−/− mice treated with PBS or B. fragilis WT-OMV during DNBS colitis. Error bars represent S.E.M. * p < 0.05, ****p < 0.0001. Two-way ANOVA, followed by Tukey’s post-hoc analysis. Data are representative of at least 3 independent experiments, with 3-5 mice/group.
Fig. 4. The T300A risk variant of ATG16L1 in human cells is unable to support OMV responses (A and B) MoDCs with either the protective (A) or risk (B) allele were treated with PBS, B. fragilis WT-OMV, ΔPSA-OMV or purified PSA, washed and co-cultured with syngeneic CD4+ T cells. IL-10 expression was analyzed by flow cytometry among CD4+Foxp3+ Tregs. Human samples were processed and analyzed in a blinded fashion. CTL, control subjects; CD, Crohn’s Disease subjects. Error bars represent S.E.M. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns, not significant. One-way ANOVA, followed by Tukey’s post-hoc analysis.