NOD2 deficiency increases retrograde transport of secretory IgA complexes in Crohn’s disease

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Intestinal microfold cells are the primary pathway for translocation of secretory IgA (SIgA)-pathogen complexes to gut-associated lymphoid tissue. Uptake of SIgA/commensals complexes is important for priming adaptive immunity in the mucosa. This study aims to explore the effect of SIgA retrograde transport of immune complexes in Crohn’s disease (CD). Here we report a significant increase of SIgA transport in CD patients with NOD2-mutation compared to CD patients without NOD2 mutation and/or healthy individuals. NOD2 has an effect in the IgA transport through human and mouse M cells by downregulating Dectin-1 and Siglec-5 expression, two receptors involved in retrograde transport. These findings define a mechanism of NOD2-mediated regulation of mucosal responses to intestinal microbiota, which is involved in CD intestinal inflammation and dysbiosis.
The ability of the host immune system to discriminate between pathogens and commensals is essential to maintain mucosal homeostasis. The critical importance of maintaining a mucosal homeostatic mechanism in the intestine is highlighted when functional or genetic deficiencies exist. An example of such failure in maintaining a finely balanced immune response is the development of chronic intestinal inflammation, such as Crohn’s disease (CD). CD is an idiopathic, chronic regional enteritis that most commonly affects the terminal ileum but has the potential to affect any part of the gastrointestinal tract from mouth to anus. CD is thought to occur as a result of a breakdown in self-recognition of commensal bacteria together with mucosal barrier dysfunction in individuals with a given genetic background. The most strongly associated genetic risk factor for CD in Western populations remains NOD2, an intracellular pattern recognition receptor important in immune defense against intracellular microbes. NOD2 is known to regulate the intestinal barrier function, limiting the transcellular permeability and bacterial translocation. The CD-associated mutations in NOD2 (Leu1007fsinsC, Gly908Arg, and Arg2021Gln), located within the LRR region of the protein, results in loss of NF-kB activation in response to muramyl dipeptide (MDP). However, the reasons why the inactivation of NOD2 can result in chronic colitis remain largely speculative.

Secretory IgA (SIgA) is the most abundant immunoglobulin on mucosal surfaces of humans and many other mammals. SIgA can protect the intestinal epithelium by discriminating commensal bacteria from enteric pathogens. Recognition of enteric pathogens by the intestinal immune system results in the production of high affinity, T-cell-dependent, pathogen-specific IgA, which is “transcytosed” into the intestinal lumen. SIgA exhibits also the striking feature to adhere to the apical membrane of M cells, promoting the uptake and delivery of antigens (Ags) to dendritic cells (DC) located in Peyer’s patches (PP). Under pathological conditions such as infection invading IgA opsonized micro-organisms, these immune complexes amplifies the production of proinflammatory cytokines such as TNF, IL-1β, and IL-23 by human CD103+DCs. This retrograde transport is called reverse transcytosis, and is mediated by epithelial M cells. Both the Cα1 domain of SIgA and its associated Sialic acid (Sia) residue glycosylation are involved in IgA reverse transcytosis, as well as Dectin-1 and Siglec-5, identified as receptors for SIgA uptake on M-cells. However, the regulation and pathway(s) whereby SIgA is retro transported across M cells still need to be elucidated.

Increase of the intestinal permeability has for many years been recognized as a pathogenic factor in CD. An abundance of clinical, epidemiologic, and animal model studies have assessed the impact of various commensal and potentially pathogenic enteric bacteria that may trigger or exacerbate IBD. The earliest, NOD2 deficiency increases IgA retro transport in mice. In the absence of NOD2, PP-associate IgA was modified as a consequence of the disease, and second refined the analysis to samples collected from CD patients with or lacking NOD2 mutations. To address this question, we first determined NOD2 genotypes from PP biopsies in the terminal ileum from healthy (n = 8) or CD patients (n = 20) followed by quantification of SIgA-positive cell numbers. The mean fold-increase of SIgA-positive cells per PP was 2.8 in CD patients expressing NOD2 frameshift polymorphisms (n = 10) compared to healthy individuals (n = 8) and CD patients expressing wild type (WT) NOD2 (n = 10). A first colocalization (Fig. 1b) with anti-IgA Ab and anti-secretory component (SC) Ab confirm that IgA was not enriched from inside (interstitial fluids) but from the lumen (retrotranslocation of SIgA from the lumen to the PP). Colocalization IgA+/SC+ was observed in all tested biopsies (healthy n = 8; CD patients with NOD2 frameshift polymorphisms n = 10; CD patients with WT NOD2 n = 10). A second colocalization IgA+/DC-SIGN+ (Fig. 1b) indicate that counted SIgA+ cells derived from the retrograde transport of SIgA through M cells followed by their DC uptake. Increase of SIgA retro transport is not observed in segments of the intestine lacking PP’s (Fig. 1a, b). Weak differences in numbers of SIgA+ cells were measured in patients with NOD2 polymorphisms R702W (n = 4) or F11007insC (n = 3) or R702W/C968R (n = 3) (Fig. 1a). The sum of the data demonstrate an increase in PP-associated SIgA in the subset of CD patients displaying mutated NOD2 gene solely.
biopsies obtained from patients with NOD2 polymorphisms, the mean fold-increase (2.3) of IgA transport from the lumen into the PP in NOD2 KO mice (112.4 ± 14.5) was significantly different when compared to WT mice (49.4 ± 7.6). This significant increase in IgA transport, together with a significant decrease after MDP-PAM treatment demonstrates the implication of NOD2 in the process of transcytosis (Fig. 2a). Finally, we did not observe any retrotranscytosis of negative controls as BSA or an irrelevant mouse IgG using a ligated intestinal loop model suggesting that the effect is IgA-dependant (Fig. 2a).

Oral administration of HIV p24 in association with IgA to littermate WT mice leads to the generation of a robust immune response against the viral antigen. In this experimental setting, IgA serves as a delivery system carrying an intact HIV p24...
Fig. 1 NOD2 mutation increases SlgA reverse transcytosis in CD patients. a The two first graph compares the frequency of IgA-positive cells between healthy donors \((n=8)\), CD WT NOD2 patients \((n=10)\) and CD patients expressing NOD2 polymorphisms \((n=10)\) in PP and in villi. The third graph shows the number of IgA positive PP cells in patients expressing different NOD2 polymorphisms. NOD2 polymorphisms \((R702W, G908R, FS1007insC)\) were determined by qPCR and sequencing. The last graph shows the ratio PP/villi. Vertical bars show the mean value ± SEM. A nonparametric Mann–Whitney \(U\)-test was used \((p\) values: **\(p<0.01\), ***\(p<0.005\)).

b Images obtained from patient biopsy samples taken from the terminal ileum. This experiment was repeated in all patients with similar results: healthy donors \((n=8)\), CD WT NOD2 patients \((n=10)\) and CD patients expressing NOD2 polymorphisms \((n=10)\). Biopsies were labeled with anti-human GFP-IgA2 and PE-GP2 to label M cells or PE-DC-SIGN or PE anti-human SC at room temperature for 2 h. The left panels show representative images from CD patients expressing NOD2 polymorphism, the middle panels, CD patients with WT NOD2, and the right panels depict images from healthy donors. Bars: 200 \(\mu m\). On all pictures, dotted lines delineate the follicle-associated epithelium (FAE) separating the intestinal lumen and the lymphoid tissue (side view). Source data are provided as a Source Data file.

Fig. 2 NOD2 deficiency increases IgA retrograde transport in mice. a Tissue section showing a PP obtained from a ligated intestinal loop in WT, NOD2 KO or WT mice after MDP-PAM stimulation following exposure to SlgA-Cy3 for 60 min. Mouse IgG and the BSA were used as negative controls. The graph shows the number of SlgA-positive cells per PP. This experiment was repeated on 6 mice per group and an average of 3 PP per mouse was done. Vertical bars show the mean value ± SEM. One-way ANOVA followed by Bonferroni post hoc test was used \((p\) values: *\(p<0.05\); **\(p<0.005\)).

b Fecal bacteria from WT or Nod2KO mice were stained with anti-IgA-FITC and the MFI were calculated on IgA-bacteria. This experiment was repeated on 4 mice per group. Vertical bars show the mean value ± SEM. A nonparametric Mann–Whitney \(U\)-test was used (ns: not significant).

c Lysed bacteria were used as the target antigens in a western blot, using serum (S) or fecal (F) supernatant from WT or NOD2 KO mice as primary antibody and anti–IgA-HRP as the secondary antibody. This experiment was repeated on eight cohoused mice (WT \((n=4)\), NOD2 KO \((n=4)\)) with similar results. NOD2 KO and littermate WT mice were cohoused and immunized orally with p24-SIgA.

d Levels of Ag-specific mucosal IgA and e levels of serum IgG in littermate WT or NOD2 KO mice were shown. Vertical bars show the mean value ± SEM. \(n=5\) biologically independent mice per group. One-way ANOVA followed by Bonferroni post hoc test was used \((p\) values: *\(p<0.05\); **\(p<0.005\)).

f Cytokine concentrations in serum and faeces were determined in triplicate by Luminex-multiplex cytokine assay in littermate WT \((n=3)\) and NOD 2 KO mice \((n=3)\). Cytokine profiles are shown as radar charts; each axis displays the mean quantity (pg/ml) of each cytokine after immunization with p24-SIgA, p24 and PBS. A nonparametric Mann–Whitney \(U\)-test was used. \(P\) values have been calculated by comparing the p24-SIgA group with the p24 group; *\(p<0.05\). Source data are provided as a Source Data file.
antigen into intestinal M cells in PPs. We thus speculated that the increased retro-transport of SigA observed in NOD2 KO mice would result in improved p24-specific reactivity after oral immunization as compared to littermate WT mice. First, we confirmed the same ability of IgA to bind to the same microbiota pattern in littermate or NOD2 KO mice, as previously described (Fig. 2c). It was also confirmed by quantification of IgA-bound bacteria with flow cytometry (Fig. 2b). As oral immunization is well known to induce both systemic and mucosal responses, the antigen-specific response was measured in both serum and feces samples 1 week after the last immunization. Systemic p24-specific IgG and fecal IgA titers were significantly increased in NOD2 KO mice as compared to littermate WT mice (Fig. 2d, e). Oral delivery of an equivalent amount of p24 alone was not sufficient to induce a specific Ab response in either KO or littermate WT animals (Fig. 2d, e). Because the local tissue-dependent nature of immune responses relies highly on the controlled production of cytokines, a panel of them was measured by Luminex in serum samples obtained 24h after the last immunization. In comparison with p24 alone, p24-SigA was a potent inducer of all the tested cytokines in serum but also TNF-α, INF-γ and IL-17 measured in feces of NOD2 KO mice (Fig. 2f). Immunization with SigA alone has no effect on the production of cytokines. Except for IL-10, no difference in cytokine secretion was observed between littermate WT and NOD2 KO mice. The increase of IL-10 in serum after immunization with p24-SigA in the NOD2 KO mice may indicate regulation of the systemic inflammatory process following initial priming of the specific Th cell response. Taken together, these results indicate that transport of p24-SigA complexes is dependent on functional NOD2 and results in the regulated passage of the hooked Ag, which is subsequently processed to trigger the onset of mucosal and systemic antibody and cytokine responses.

SigA-Salmonella amplify Salmonella-induced colitis in mice. The demonstration that SigA reverse transcytosis in NOD2 KO mice favors the transport of a bound Ag, as reflected by the detection of specific immune responses, suggests that such a mechanism may be involved in the pathogenesis of chronic colitis. To address this hypothesis, KO mice displaying opposed effects on intestinal SigA retrograde transport (i.e., NOD2 KO mice identified as promoting intestinal retrograde transport (this study), and Dectin-1 KO mice known to abrogate reverse transcytosis) were used and compared with WT littermate animals. The disease condition was triggered upon administration of dextran sodium sulfate (DSS) in drinking water (positive control), or after oral delivery of Salmonella Typhimurium alone (Salmonella-induced colitis model) or in association with murine Salmonella-specific SigA-Sal4 (working hypothesis). In control experiments, oral administration of the Dectin-1 antagonist laminarin, known to suppress the development of DSS-colitis, was used to further verify the impact of SigA retrograde transport inhibition.

In comparison with the PBS control group, oral administration of Salmonella alone in littermate WT mice resulted in a significant increase of inflammation severity, as determined by the measurement of the disease activity index (DAI) (Fig. 3a). Oral delivery of Salmonella-SigA-Sal4 complexes led to a weak, yet statistically significant further increase in DAI at day 5 (Fig. 3a). DSS-treated littermate WT mice showed the highest signs of disease. Blocking of Dectin-1, and thus reduction of SigA reverse transcytosis by addition of laminarin in the drinking water, markedly decreased the severity of colitis. In support of our working hypothesis, NOD2 KO mice with increased SigA reverse transcytosis and thus higher intestinal transport of Salmonella exhibited a DAI resembling that of DSS-treated animals (Fig. 3a), well above the index observed in the Salmonella alone condition. Such a colitis-preventing effect was impaired upon delivery of laminarin to mice (Fig. 3a). The “clinical” progression of colitis was also confirmed by quantifying neutrophil infiltration in the lamina propria (Fig. 3b), according to the Nancy histological score (Fig. 3c), by measuring weight loss (Supplementary Fig. 1a), serum IL-6, CRP, and Lipopolysaccharide (LPS) (Supplementary Fig. 1b). The high level of blood LPS in Salmonella-IgA treated mice reflects the IgA-based retrotranscytosis of Salmonella through the intestinal epithelium. This seems to demonstrate the inability of Sal4 IgA to neutralize Salmonella under our experimental conditions as previously described. To confirm this point, we measured the aggregation of Salmonella Typhimurium after SigA-Sal4 binding. We did not observe in vitro a significant increase in Salmonella aggregates after SigA binding (Supplementary Fig. 1c). The absence of aggregates confirms the non-neutralizing character of Sal4 IgA in our experimental conditions. This could indicate that these aggregates are only capable of forming in vivo and do not alter the retrotranscytosis ability of IgA.

SigA-Salmonella treated mice without laminarin have been isolated, plotted and significantly highlighted by comparing littermate WT and Nod2KO mice (Supplementary Fig. 1d). The significant difference of DAI at day 5 after SigA-Salmonella administration clearly confirms the role of Nod2 in SigA-Salmonella transport. This statement was also observed using a Salmonella-GFP bound with SigA administered into a ligated intestinal loop containing a PP in NOD2 KO mice and WT littermate mice (Supplementary Fig. 1e). Results obtained by blocking Dectin-1 by the addition of laminarin in the drinking water were similar by using Dectin-1 KO mice (Supplementary Fig. 1f) and are consistent with the previous reports. Thus, SigA reverse transcytosis seems to play a role in colitis induction by a NOD2-mediated increased transport of pathogens.

NOD2 modulates the Dectin-1 and Siglec-5 expression. In the mouse intestine, selective IgA reverse transcytosis across the epithelium has been shown to depend on the expression of Dectin-1 and to a lesser extent Siglec-5 on M cells. IgA reverse transcytosis can be recapitulated in the in vitro model of FAE comprising human polarized Caco-2 cells and M-like cells. Traditional transport (transcytosis) across polarized epithelial of serosal polymeric IgA into secretions requires polymeric Ig receptor and a plethora of evolutionary well-conserved intracellular proteins including EEA-1, Rab-5, Rab-9, Rab-11, Rab-17, and Rab-25. Because the regulation of vesicle trafficking along the retro-transcytotic pathway is unknown, we found it important to determine whether NOD2 may somehow contribute to fine-tune the expression of either one of the two receptors and/or the putative organelle-specific proteins.

In order to address the nature of proteins possibly involved, we took advantage of the in vitro model of FAE. In the absence of M-like cell conversion, the plain Caco-2 cell polarized monolayer (mono-culture) allowed weak retrotranscytosis of the reporter IgA-luciferase fusion protein (IgA-Luc), in agreement with previous data. This background level was not affected after transfection with siRNA targeting a selection of extra- and intracellular proteins, as indicated in Fig. 4a. In the presence of interspersed M-like cells in the polarized monolayer (co-culture), SigA reverse transcytosis of IgA-Luc was increased by a factor of 4 (Fig. 4a, lanes control and random non targeting oligo). Among the candidate assayed, targeting of EE-1, Rab-5, Rab-17, as well as SYK and TAK1 proteins involved in Dectin-1 signaling pathway led to a significant 3-fold decrease of SigA-Luc
transport, as compared to controls (Fig. 4a). This decrease compares with that resulting from the targeting of Dectin-1 and Siglec-5 used as positive controls. A significant twofold increase of IgA transport in cells treated with NOD2 siRNA, together with a significant threefold decrease after MDP-PAM treatment confirm the implication of NOD2 in the process of transcytosis, and indirectly validates the results obtained via the FAE model (Fig. 4a). Western Blot (Supplementary Fig. 2a) or quantitative RT-PCR was performed to correlate the decrease of mRNA level and its expected increase after MDP/PAM treatment, (Supplementary Fig. 2b). In additional control experiments, both the decrease in the level of targeted proteins and the monolayer integrity (TEER) were systematically monitored after siRNA transfection (Supplementary Fig. 2c). No significant modifications between controls, knockdown and stimulated cells were observed. Staining of tight junctions using ZO-1 detection confirmed the monolayer integrity after siRNA transfection (Supplementary Fig. 2d). Finally, the retro transport of cholera toxin (CT), which is known to use the Rab-5 pathway36, has been using as a positive apical-to-basal transport control to test the efficacy of siRNA knockdown on either monoculture or coculture conditions (Supplementary Fig. 2e). After Rab-5 siRNA knockdown, CT transport is significantly reduced in our inverted in vitro model, confirming the role of Rab-5 in CT transport and the efficacy of siRNA knockdown.

To identify if EEA-1, Rab-5, Rab-17, Dectin-1, and Siglec-5 are key partners in IgA reverse transcytosis, IgA2 was added apically in the in vitro model of FAE for 30 or 60 min, and IgA2-associated proteins complexes were recovered after immunoprecipitation with protein M-agarose beads. Association between IgA2 and EEA-1, Rab-5, Rab-17, Dectin-1, and Siglec-5 was revealed by immunodetection with a battery of specific antibodies (Fig. 4b). Specific colocalizations IgA2+/EEA-1+, IgA2+/Rab-5+, and IgA2+/Rab-17+ were also confirmed by immunofluorescence. No colocalization was observed with Rab-7, Rab-9, Rab-11, Rab-25, and pIgR with IgA (Fig. 4c).

The role of NOD2 in the modulation of protein expression associated with SIgA reverse transcytosis was investigated next. The in vitro model containing M-like cells (co-culture) or not (mono-culture) was used as such, or following of NOD2 knockdown with siRNA. Proteins were recovered from whole cell lysates and their relative abundance was assessed by Western blot (Fig. 5a). Specific colocalizations IgA2+/EEA-1+, IgA2+/Rab-5+, and IgA2+/Rab-17+ were also confirmed by immunofluorescence. No colocalization was observed with Rab-7, Rab-9, Rab-11, Rab-25, and pIgR with IgA (Fig. 4c).

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relevant changes were observed upon analysis of the endosomal proteins tested. MDP-PAM treatment led to a decrease of the expression of the two IgA receptors (Fig. 5b). When examining specifically the expression of Dectin-1 and Siglec-5 in GP2+ M-like cells, as tested by flow cytometry, a consistent threefold increase of either receptor that occurred in NOD2 knockdown cells was confirmed as compared to unconverted cells with an enterocyte phenotype (Fig. 5c). In support of data in Fig. 5b, MDP-PAM treatment did not result in any significant changes in the expression of either receptor. Dectin-1 and/or Siglec-5 expression has not been detected on enterocytes. This observation has been confirmed in NOD2 KO mice where Dectin-1 expression is increased on PP M cells compared to WT mice (Fig. 5d). Finally, we investigate the involvement of NOD2 in other stages of the mucosal IgA pathway, such as production. Figure 5e clearly shows no significant difference in IgA concentration observed in serum or feces between NOD2KO mice and littermate WT mice. The sum of the data implicates NOD2 in M-like cells as the plausible player involved in Dectin-1 and Siglec-5-mediated facilitated retrograde transport of IgA across a tight epithelial mimic.

Discussion

So far, the mechanisms involved in the loss-of-function polymorphisms on downstream NOD2 signaling and the pathogenesis of CD remain largely unknown. A commonly recognized finding is that defects in NOD2 result in a constitutively weak inflammatory response that can lead to increased intestinal bacterial load and with time to chronic intestinal inflammation observed in CD. Here we demonstrate that the mucosal inflammation observed in CD patients is could be due to increased transport through M cells of IgA-bacteria complexes from the lumen to immune cells present in the PP. First, we found that IgA reverse transcytosis was significantly increased through human (Fig. 1) and mouse (Fig. 2) M cells when NOD2 is mutated or absent. At a steady-state, NOD2 seems to down-regulate the IgA retrograde transcytosis through M cells. In CD or colitis mouse models, the
absence of NOD2 could increase the transport of IgA-bacteria complexes inducing mucosal inflammation. It is well established that the polymorphism of the NOD2 gene is a major risk factor in CD. However, a molecular explanation of how such loss of function leads to increased susceptibility to CD remains unclear. Hedl et al.38 have shown that NOD2 signaling activates the mTOR pathway which induces the upregulation of anti-inflammatory mediators and simultaneously the downregulation of pro-inflammatory cytokines. These data support the idea that NOD2 modulates innate immune responses to intestinal microbiota and thus suggest that the absence of such regulation leads to increased susceptibility to CD.

The role of the IgA reverse transcytosis in the pathogenesis of chronic colitis was identified in Dectin-1 KO mice and littermate WT mice using the well-known Salmonella colitis model39,40. Dectin-1 signaling has been described to regulate intestinal inflammation by controlling commensal Lactobacillus-mediated colonic regulatory T cells34. It seems that the transport of IgA-bacteria complexes through M cells via Dectin-1 receptor19 induces intestinal inflammation, suggesting some degrees of correlation between the abundance of M cells and chronic intestinal inflammation. In this respect, Bennet et al.41 found that in both the dextran sodium sulfate and Citrobacter rodentium models of colitis, significantly increased numbers of PP M cells were induced at the peak of inflammation in the colonic epithelium. Using KO mice, we demonstrate that NOD2 acts as an immune regulator of IgA reverse transcytosis. Indeed, NOD2 KO mice administered with Salmonella-IgA complexes exhibit an increase of the inflammation’s severity as compared to littermate WT mice. This observation is supported by studies showing that NOD2 can modulate inflammation and mediate efficient clearance of bacteria from the mucosal tissue during Salmonella colitis42,43.

We next investigated the molecular mechanisms which could explain how NOD2 modulates IgA retrograde transport. Using a specific siRNA knockdown approach in the in vitro model containing M-like cells, the involvement of NOD2 as a regulator of IgA transport was confirmed. The role of endosomal proteins already known to be involved in the IgA transport through enterocytes was also studied44. Our data reveal that IgA reverse
transcytosis through M cells seems to be mediated by EEA-1, Rab-5, and Rab-17 endosomal proteins as it has been previously described for epithelial IgA transcytosis. The role of Dectin-1 and Siglec-5 in IgA reverse transcytosis was also confirmed. NOD2 has also been shown to influence MHC cross-presentation, autophagy induction, and resistance to intracellular bacterial infection. Thus, while principally well-known for its acute signaling effects, NOD2 activation causes a variety of cellular changes in vivo that are also likely important for immune homeostasis.

SYK and TAK1 proteins from the Dectin-1 signaling pathway are also involved in the blocking of IgA reverse transcytosis. The role of NOD2 interaction with TAK1 through its leucine-rich repeat (LRR) region to exert its inhibitory effect on TAK1-induced NF-κB activation has been published. This suggests that NOD2 inhibits TAK1-induced NF-κB activation, which results in the downregulation of IgA transport. A significant decrease of Dectin-1 and Siglec-5 expression after NOD2 knockdown in M cells is observed in the current study, suggesting that NOD2 contributes to regulate the expression of these two receptors for SIgA.

Our finding should be interpreted with caution, as the etiology of CD involves a combination of genetic, environmental, and microbial factors. Hence, we find it fair to underline that the SIgA-dependent route identified in the study is likely to be one among other mechanisms that eventually initiates and/or perpetuates mucosal inflammation observed in CD patients. However, our data confirm and extend the knowledge that the NOD2 genotype status is currently the strongest genetic marker associated with a severe CD course. In Fig. 1, NOD2 mutations of the studied population (R702W, 1007fs and R702W/G908R) were compared and no differences were observed in IgA-positive cells distribution in PP. These results are consistent with the study of Hugot et al., which provided strong evidence that the penetrance of the most at-risk genotypes is low. They found no clear relationship between mutation frequencies and the disease incidence in their studied populations and no significant deficit of double-dose mutation carriers among healthy controls. Nevertheless, these two studies confirm that CARD15/NOD2 acts in interaction with other unknown risk cofactors.

A theory proposes that antibodies generated in response to microbial colonization of the intestine shape the microbiota composition to benefit the health of the host through a process called antibody-mediated immunoselection (AMIS). Immuno-selection refers to a process of natural selection within a host organism that is mediated by the immune system to influence microbial fitness and hence microbial ecology and evolution. A significant fraction of commensal bacteria is heavily coated with IgAs. However, such coating seems to be non-specific, as there is a significant overlap of bacterial species between IgA-coated and non-coated fractions. Under such conditions, the bacteria diversity was significantly reduced. Thus, it seems that reduced affinity maturation of IgAs is associated with reduced diversity and skewed microbiota and abundant coating of bacteria with natural IgAs. A recent paper goes further by saying that bacteria recognized by human SIgM were dually coated by SIgA and showed increased richness and diversity compared to IgA-only-coated or uncoated bacteria. This bacterial selection mechanism could be a causal agent for CD development or other

Fig. 6 The retrograde transport of IgA-pathogen complexes across M cells is increased in CD patients as compared to healthy individuals. This translocation of IgA is regulated by WT NOD2 upon decrease of Dectin-1 and Siglec-5 expression in M cells, ensuring proper homeostasis. This pathway is controlled by endosomal proteins such as EEA-1, Rab-5, and Rab-17. We would like to emphasize that this identified mechanism is likely one among others that is involved in the initiation and/or perpetuation of mucosal inflammation observed in CD patients.
IgA-based diseases where IgA reverse transcytosis could play a role. Indeed, CD patients have several features in common with IgA nephropathy and celiac disease. Increased small intestinal mucosal permeability has been demonstrated in these pathologies. In IgA nephropathy, this may allow the influx of food and bacterial Ags resulting in immune complex formation and deposition. In celiac disease, the alteration of para- and transcellular pathways has been proposed to explain the retrograde transport of intact peptides, and notably the apico-to-basolateral translocation of SlgA ensured by the transferrin receptor.

A better understanding of molecular mechanisms driving chronic gut inflammation has led over the past two decades to therapeutic strategies with major impacts for the current management of IBD. However, despite spectacular successes, mainly attributable to the anti-TNF therapy, not all patients respond to the drugs and about one third of the responders relapse within a short period of time. Further works are therefore needed to identify therapeutic molecules in IBD. In the present study, we demonstrated that IgA retro-transport is involved in promoting inflammation in CD by adopting the transport of IgA-bacteria immune complexes in the PP through M cells. Therapeutic strategies aiming at blocking IgA reverse transcytosis during the acute phase of CD may be considered to design new efficient immunotherapeutic strategies.

### Methods

**Immunolabeling of human PP ex vivo.** Informed and consenting CD patients or healthy individuals who had undergone lower endoscopy for routine diagnostic purposes with normal intestinal mucosa provided two biopsy samples from the terminal ileum. Biopsies were fixed for 2 h in 3% paraformaldehyde and included in OCT embedding solution, before crossectioning with a Leica cryostat model CM1950. Ten-micrometers sections were captured on UltraPlus Super frost microslide slides and stained for M cells. Slides were washed in PBS to eliminate residual OCT embedding solution, and blocked with PBS containing 5% FBS for 30 min at room temperature. Immunolabeling was performed using a combination of GFP-IgA2 (Invivogen), anti-human PE-GRP mAb (MBL), anti-human PE-DG-SIGN mAb (Thermo/isher scientific), and mouse anti-IgA secretory component Ab (Abcam) followed by goat anti-mouse PE-IgG (Abcam) diluted to 20 µg/ml for 2 h at room temperature. The slides were then washed in PBS, air-dried, and mounted with Fluoprep (Biomerieux). Slides were observed by immunofluorescence microscopy (Eclipse, Nikon).

**Oligonucleotide sequences for NOD2 polymorphism PCR.** PCR of NOD2 polymorphisms R702W: forward, 5′-CTG AGC CTT TGT TGA TGA GC-3′; reverse, 5′-CTG AGC CTT TGT TGA TGA GC-3′. HIV-1 capsid protein from clade B strain (Px

**Mice.** NOD2 KO mice were obtained from Gabriel Nunez (University of Michigan, USA). Dectin-1 KO mice were obtained from Gordon D. Brown (University of Aberdeen, UK). NOD2 KO mice and Dectin-1 KO mice were described. Littermate mice were obtained from NOD2-heterozygous crosses. All mice were born and housed at the PLEXAN (Platform for Experiments and Analysis, Faculty of Medicine, Université de Saint-Etienne, France) which is a conventional animal facility with infectious sector P2. All mice were housed in the same conditions (Temperature of 20 °C with 50% humidity, 12 light/12 dark cycle, unlimited access to food and water), and were females between 2- and 4-months old. The experimental protocols have been approved by the French ministry of research, the local ethical committee (CEEA-Loire) and the Animal Welfare Committee of the PLEXAN (agreement no. 201701131536174_v4). SlgA administration into ligated loops. For ileal loop preparation, mice were anesthetized by an intraperitoneal injection of a mix of ketamine and xylazine (100 and 10 mg/kg animal weight, respectively) and kept warm at 37 °C throughout the surgical procedure. Hundred microliters of a 1 mg/ml solution of SlgA-Cy3 diluted in PBS or Salmonella(GFP)-SlgA4 complex immune complexes (see a model of mouse colitis, BSA (Sigma), or mouse IgG (anti-human TAK1 mAb (R&D Systems)) were administered into a 1-cm ligated loop containing a P. salmonellae entérica subsp. enterica serovar Typhimurium GFP were obtained from ATCC (14028GFP). Upon completion of the experiment, the mice were sacrificed by cervical dislocation and the piece of intestine was removed, extensively washed with PBS, fixed for 2 h in 3% paraformaldehyde, and included in optimal cutting temperature compound (OCT) embedding solution. Sections (5 µm) were collected on UltraPlus Super frost microslide slides (VWR International). Slides were observed by immunofluorescence microscopy (Eclipse, Nikon). The protocol followed the guidance of the regional Ethics Committee for animal testing, CREEA (Permit number: No. 69387487).

To measure mouse SlgA retrotranscytosis, a polymeric IgA Ab (clone IAC5 specific to S. flexneri serotype 2A SPS)67 was obtained as previously described. Purified free human SC was produced in Chinese Hamster Ovary cells66. SlgA was obtained by mixing in PBS pIgA molecules with a twofold excess of human SC for 2 h at room temperature according to the conditions described previously.37-C3 SlgA complexes were obtained by conjugation with indocarboxyanine (Cy3) using the FluoroLink mAb Cy3 labeling kit (Amersham Biosciences).

Study of IgA/fecal microbiota interactions. The ability of fecal microbiota to bind gut microbiota has been measured by western blot63. Lyzed bacteria were used as the target antigens in a western blot assay. Serum or fecal supernatant from WT or Nod2KO mice were used as primary antibody and anti-IgA-HRP as secondary antibody. This experiment was repeated on 4 mice per group. The quantity of IgA already bound with fecal bacteria were also quantified by flow cytometry. Fecal supernatant from WT and Nod2KO mice were stained with anti-IgA-HR and the MFI were calculated for IgA. This experiment was analysed using 4 mice per group.

In vivo delivery of p24-SlgA. HIV-1 p24 capsid protein from clade B strain (Px Therapeutics, France) was covalently associated with polymeric SlgA65 using the Sulfo-KMUS heterobifunctional crosslinker (Thermo Scientific). Covalent complex formation was verified by Western Blot. p24-HIV-1 serum was purified on protein G and revealed with anti-human IgG HRP-conjugated secondary Ab (Amersham). Mouse oral immunizations were performed by orogastric intubation with polylactide tubing under light anesthesia with isoflurane (Halocarbon Laboratories). The tubing was introduced at a fixed distance of 1.8 cm from the incisors. Immunizations consisted of three administrations of 100 µl at 1-week intervals. Littermate WT (n = 5), and Nod2KO (n = 5) mice were immunized with 100 µg of HIVp24-SlgA, or SlgA alone, or HIVp24 alone per administration.

### Measurement of HIVp24-specific IgG and IgA Abs. Serum and feces samples were recovered 1 week after the last immunization. Five fresh feces were collected from each animal. Feces were incubated with Halt Protease Inhibitor Cocktail (Thermo Scientific), centrifuged at 16,000 g and stored at −20 °C until use. Specific IgG against HIVp24 were measured using a quantitative ELISA. Maxisorp 96-well plates were coated with either 50 µl of HIV p24 Ag solution (5 µg/ml in sterile PBS) or 50 µl of a 1/3200 dilution of an equal mixture of anti-mouse Ig kappa and lambda light chain-specific mAbs (Serotec), and then incubated ON at 4 °C. Murine IgG or IgA immunoglobulins (Igs) (Southern Biotech) were used as standards. Bound or captured IgG was detected by incubation with HRP-conjugated goat anti-mouse IgG (IgG), while IgA was detected using biotinylated goat antianti-mouse IgA (Southern Biotech) followed by streptavidin-HRP (Amersham). Results are given as the means of concentrations ±SEM.

### Cytokines and chemokines. The evaluation of multiple cytokines/chemokines was performed with a Luminex 100 instrument (Luminex Corporation, Austin, TX, USA), in combination with the Bio-Plex mouse cytokine 23-plex panel and Bio-Plex mouse cytokine Th17 panel B-8-Plex Group III (Bio-Rad, Berkeley, CA, USA). Biological fluids were recovered 1 week after the last immunization. Cytokine and chemokine concentrations were determined as the mean of three replicates.

### Model of mouse colitis. The virulent streptocin-resistant Salmonella enterica serovar Typhimurium strain SL1344 was cultured in LB (LB Broth, Sigma) supplemented with 100 µg/ml of streptomycin (LB-St). A day before infection, a SL1344 colony was cultured overnight at 37 °C, 100 rpm in 3 ml of LB-St. Mouse IgA4, specific for Salmonella Typhimurium surface carbohydrates was produced as described previously and used to treat mice with SlgA-Salmonella complexes. In other experiments, colitis was also induced by giving 5% dextran sodium sulfate solution in drinking water ad libitum for 7 days68. Body weight and DAI were monitored each day.

**NO2 KO (n = 5), Dectin-1 KO (n = 5), and littermate WT (n = 5) mice were given either Salmonella-SlgA4 complexes or Salmonella alone (i.e., 106 CFU/mouse) in 100 µl PBS by orogastric intubation with polylactide tubing under light anesthesia with isoflurane (Halocarbon Laboratories). A day before infection, a SL1344 colony was cultured at overnight at 37 °C, 100 rpm in 3 ml of LB-St. Mouse IgA4, specific for Salmonella Typhimurium surface carbohydrates was produced as described previously and used to treat mice with SlgA-Salmonella complexes. In other experiments, colitis was also induced by giving 5% dextran sodium sulfate solution in drinking water ad libitum for 7 days68. Body weight and DAI were monitored each day.

**Induction of colitis in NOD2 KO mice**. NOD2 KO mice were either Salmonella-SlgA4 controls or Salmonella alone (i.e., 106 CFU/mouse) in 100 µl PBS by orogastric intubation with polylactide tubing under light anesthesia with isoflurane (Halocarbon Laboratories). NOD2 KO mice were first infected with a Salmonella SL1344 strain that was cultured at overnight at 37 °C, 100 rpm in 3 ml of LB-St. Mouse IgA4, specific for Salmonella Typhimurium surface carbohydrates was produced as described previously and used to treat mice with Salmonella-Salmonella complexes. In other experiments, colitis was also induced by giving 5% dextran sodium sulfate solution in drinking water ad libitum for 7 days68. Body weight and DAI were monitored each day.
Assessment of colitis severity. During the duration of the experiment, the DAI score was monitored daily to evaluate the clinical progression of colitis\(^9\). The DAI score combines read-outs including: weight loss compared to initial weight, stool consistency, and rectal bleeding. Scores were de

bound proteins) were concentrated by immunoprecipitation with protein M-agarose beads (Invivogen). Post washes, elution was performed with 0.1 M glycine (pH 3.0), followed by an elution buffer containing 1 M Tris buffer (pH 8.0). The eluted material was subjected to SDS-PAGE followed by transfer onto a hybird ECL nitrocellulose membrane (GE Healthcare Life Science). Immunodetection of targeted proteins was performed with a selection of Abs/antisera including: Goat anti-human Dectin-1/CLEC7A 7A serum, anti-human CD170 (Siglec-5) mAb (mouse IgG1, Clone #914128), anti-human EEA-1 serum, anti-human plgR mAb (Mouse IgG2, Clone # 825274), anti-human SYK mAb (mouse IgG1, Clone # 720402), anti-human TAK1 mAb (mouse IgG1, Clone # 918410) were purchased from Bio-Rad. Appropriate HRP-coupled secondary Abs were used for detection with the "Clarity Western ECL Substrate" (Biorad).

Immunofluorescence staining. Ten microliters of IgA2 (Invivogen) were added to the apical compartment of the in vitro model of FAE. The cells were incubated for 60 min at 37 °C. Incubation with 0.1% Triton X-100 Sigma-Aldrich, followed by blocking with 5% FBS for 15 min at room temperature. Immunolabeled was performed using a combination of same antibodies and anti-EA-1 Abs described in the previous section. This reagent was diluted to 1/100 and incubated for 2 h at room temperature. Corresponding secondary antibodies labeled with a PE were incubated for 1 h at room temperature. After two washes, inserts were air-dried, mounted with Fluoprep (BioMerieux), and observed by Immunofluorescence microscopy (Eclipse Ti, Nikon).

NOD2 RT-PCR\(^5\) Total RNA was extracted using TRIZOL (Invitrogen). Reverse transcription was performed using the PrimeScript RT reagent kit (TaKaRa Bio-technology, Dalian, PRC). The SYBR Premix Ex Taq" II kit (TaKaRa Bio-technology) was used to amplify GAPDH and NOD2 gene products. The oligonucleotide primers used were as follows: NOD2: forward, 5'TCG AAT AAC GGC CCC AAG GT -3' and reverse, 5'GTC TCT TGG AGG CAC GGG ATG ATG-3'; GAPDH: forward, 5'TGC ACC ACC AAC TCA GTG TG-3' and reverse, 5'GCC ATG GAC TGT GGT CAT GAG-3'. The double standard curve method was used to analyze the relative gene expression.\(^6\)

IGA-Salmonella agglutination assay. Salmonella agglutination assay was performed by IgASal4 antibodies. Unrelated IgA hybridoma supernatant (anti-V. cholerae) or µg was added to 0.1 ml of an overnight culture of bacteria and incubated in round-bottom ELISA plates. The agglutination endpoint was reached when mice exhibited weight loss >20% of initial weight with dehydration and diarrhoea had to be euthanized by cervical dislocation following inhalation of isoflurane.

To evaluate histological damages reflecting colitis severity, a small fragment (0.5-1 cm) of the colon was fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin/eosin using the published procedures\(^70\). Scoring of neutrophil infiltration was performed using the Nanny histological score which has been evaluated by a blinded pathologist.

Mice were bled 5 days after treatment through retro-orbital plexus. The presence of IL-6, LPS, and CRP in sera was assessed by ELISA (Mouse IL-6 ELISA MAX, Biologend, San Diego, CA; Mouse Lipopolysaccharide ELISA Kit, ELISagenie, London, UK; Mouse C-Reactive Protein/CRP DuoSet ELISA, R&D system, USA).

In vitro model of the human FAE\(^9,73\). Inverted Transwell polycarbonate inserts (12 wells, pore diameter of 3.0 µm, Corning) were coated with Matrigel\(^7\), a basement membrane matrix (BD Biosciences) prepared in pure DMEM to a final protein concentration of 100 µg/ml for 1 h at room temperature. The coating solution was removed and inverted inserts were washed with 300 µl of DMEM. Caco-2 cells (3×10⁴), suspended in 300 µl of complete DMEM, were seeded on the lower insert side and cultured overnight. The inserts were then inverted and placed in a 12well culture dish and kept for 9 days. Raji B cells (5×10⁴) were resuspended in complete DMEM and then added to the basolateral compartment of the Caco-2 cells, and cocultures were maintained for 5 days. Mono-cultures of Caco-2 cells, cultivated as above but without addition of Raji B cells, were used as controls. The establishment of polarized co- and mono-cultures was controlled by measurement of TEER using an EndohmTM tissue resistance chamber (Endohm-12, World Precision Instruments) connected to a Milllicell-ERS Ohmmeter (Millipore). The mean TEER value of medium alone (9±0.4 Ω·cm²) was subtracted from each measurement. In random samples, the tight junction proteins were confirmed by monoclonal antibodies: occludins-1 (ZO-1) and claudins-2 (ZO-1) immunolabeling\(^8\). For transcytosis analyses, inserts were inverted prior to incubation in a 6-well plate, and a piece of silicon tubing (14 (height)×20 (diameter) mm, Labomodere) serving as a medium reservoir was placed on the surface facing the basolateral pole of the cell monolayer.

Gene inhibition by small interfering (si) RNA. Cells in the inverted vitro model of FAE were transfected at a final concentration of 5 nM with ON-TARGETplus SMARTpool siRNAs (Dharmacon) using Silentfect reagent (Bio-Rad) according to the procedure provided by the manufacturer. The reference numbers for gene targeting were as follows: Dectin-1: L-012476-00-0005; Siglec-5: L-011519-00-0005; NOD2: L-004102-00-0005; IgA: L-004102-00-0005; pIgR: L-004009-00-0005; Rab-25: L-004102-00-0005; Rab-7: L-004011-00-0005; Rab-9: L-004116-00-0005; Rab-5: L-004002-00-0005; Rab-25: L-010366-00-0005; Syk: L-003170-00-0005; NOD2: L-003464-00-0005; TAK-1: L-003464-00-0005; TAK-1 was silenced by using sequence 63175 (Cell Signaling Technology).
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Author contributions
N.R., E.M., R.G., B.C., and F.J. carried out the experiment. N.R. wrote the manuscript with support from X.R., B.C., and S.P. B.C. and S.P. supervised the project.

Competing interests
The authors declared no competing interests.

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