Tolerance to innocuous antigens from the diet and the commensal microbiota is a fundamental process essential to health. Why tolerance is efficiently induced to substances arising from the hostile environment of the gut lumen is incompletely understood but may be related to how these antigens are encountered by the immune system. We observed that goblet cell associated antigen passages (GAPs), but not other pathways of luminal antigen capture, correlated with the acquisition of luminal substances by lamina propria (LP) antigen presenting cells (APCs) and with the sites of tolerance induction to luminal antigens. Strikingly this role extended beyond antigen delivery. The GAP function of goblet cells facilitated maintenance of pre-existing LP T regulatory cells (Tregs), imprinting LP dendritic cells with tolerogenic properties, and facilitating LP macrophages to produce the immunomodulatory cytokine IL-10. Moreover, tolerance to dietary antigen was impaired in the absence of GAPs. Thus, by delivering luminal antigens, maintaining pre-existing LP Tregs, and imprinting tolerogenic properties on LP-APCs GAPs support tolerance to substances encountered in the hostile environment of the gut lumen.

Mucosal Immunology (2020) 13:271–282; https://doi.org/10.1038/s41385-019-0240-7

INTRODUCTION
The single layer epithelium lining the gastrointestinal (GI) tract is the interface between the host and the luminal environment containing trillions of microbes. At this site, the immune system encounters an array of foreign substances, ranging from innocuous dietary antigens and commensal microbes to pathogens. Responding appropriately to each of these is critical to maintaining immune homeostasis in this potentially hostile environment. Paradoxical to the hostile environment of the gut lumen, steady state encounters with non-pathogenic antigens originating from this site result in the induction of antigen specific tolerance, which is largely mediated by CD4+ Foxp3+ T regulatory cells (Tregs). 1 Despite advances in our understanding of mechanisms inducing antigen specific Tregs, why tolerance is so efficiently induced to antigens originating from this particularly hostile environment of the gut lumen remains incompletely understood.

The intestinal lamina propria (LP) contains an array of antigen presenting cells (APCs), including classical CD103+ CD11b− IRF8 dependent dendritic cells (DCs), IFR4 dependent CD103+ CD11b+ DCs, and CD103− CD11b+ cells that can express IRF4 and can include resident macrophages, with the CD103+ CD11b+ and CD103− CD11b+ APCs making of the majority of the population in the LP. 2–7 Collectively these cellular populations, excluding B lymphocytes, will be referred to as LP-APCs. While each subset preferentially supports various phenotypes of antigen specific T cell responses, there is an evolving understanding that they may play redundant roles in the induction of oral tolerance. 8 While tolerogenic responses can be induced in Peyer’s Patches and potentially in other mucosal lymphoid tissues, it has become appreciated that the gut draining lymph nodes are critical sites for the induction of oral tolerance. 9,10 Current understanding is that this process requires the acquisition of antigens by LP-APCs underling the villous epithelium, their trafficking to the draining lymph nodes to induce naive CD4+ T cells to differentiate into peripherally induced Tregs (pTregs), and homing of these pTregs to the LP where they are maintained by continued stimulation by LP-APCs acquiring the cognate antigen for these pTregs from the lumen. 11,12 Tolerance to luminal antigens occurs in the small intestine (SI) 13 and in the distal colon, 14 indicating these are the sites where luminal antigens cross the epithelium and are acquired by LP-APCs. How antigens are captured by LP-APCs at these sites may be the basis for why tolerance is effectively induced in this hostile setting.

Several routes by which luminal substances cross the epithelium have been identified including paracellular leak, the direct capture by LP-APCs via expansion of trans-epithelial dendrites (TEDs) into the gut lumen, passage from the lumen via villous M cells, and passage from the lumen via goblet cell associated antigen passages (GAPs). 15–22 Of these, LP-APC extension of TEDs is the currently favored route to support the induction and maintenance of tolerance to luminal substances in the steady state, as the extension of TEDs does not compromise the epithelial barrier and would allow direct acquisition of luminal antigens by LP-APCs. 16 However, this process directly exposes the LP-APCs to luminal contents, which in vitro studies indicate induces mixed Th1 and Th2 responses. 23 In addition, TEDs are absent in some mouse strains, which do not display defects in oral tolerance 24 and are lacking in regions of the gut where gavaged antigen is...
captured by LP-APCs\textsuperscript{25,26} suggesting that other luminal antigen acquisition pathways could support oral tolerance. Thus, how luminal antigens are acquired by LP-APCs for the induction of tolerance and if this process is integral to efficiently inducing tolerance in the hostile gut luminal environment remain unclear. Here we evaluated steady state routes of luminal antigen capture by LP-APCs. We found that LP-APC extension of TEDs, villous M cells and paracellular leak did not correlate with effective antigen capture by LP-APCs. In contrast the density of GAPs directly correlated with LP-APC luminal antigen capture and with the regions within the gut where tolerance is induced to luminal substances. Moreover, beyond the role of antigen delivery, we found that the GAP function of goblet cell imprints and maintains LP-DCs and macrophores with tolerogenic properties, maintains pre-existing Tregs in the SI LP, and in the absence of GAPs tolerance to a dietary antigens is impaired. Thus, the GAP function of goblet cells acts as both a pathway to deliver luminal substances to LP-APCs and as a mechanism imprinting LP-APCs with tolerogenic properties to maintain and induce tolerance to antigens encountered in the hostile environment of the gut lumen.

RESULTS

The presence of goblet cell associated antigen passages (GAPs), but not LP-APC extension of TEDs or villous M cells, correlates with the sites of luminal antigen capture for the induction of tolerance in the steady state, tolerance to luminal substances is induced in the SI and distal colon.\textsuperscript{13,14} How luminal substances cross the epithelium to be encountered by the immune system is a fundamental process that may underlie why tolerance is so efficiently induced to substances arising from an unfavorable environment with abundant microbes and microbial products. To evaluate how dietary antigen traverses the intestinal epithelium we performed intraluminal injections of fluorescently labeled ovalbumin (Ova) and evaluated fixed intestinal sections by fluorescent microscopy. Immuno-fluorescent staining of fixed tissue sections demonstrated that goblet cells containing the luminaly administered fluorescent Ova could be identified throughout the SI and in the distal descending colon and sigmoid colon, referred to as the distal colon, but were less common in the cecum, ascending colon, transverse colon, and proximal descending colon, referred to as the proximal colon (Fig. 1a–d). The presence of GAPs in the distal colon was not appreciated in the work initially identifying GAPs using the in vivo imaging approach due to the difficulty of imaging the distal colon with this approach. This regional distribution of GAPs correlates with the previously identified lymph nodes draining the regions of the gut supporting tolerance.\textsuperscript{13,14} Secretory intestinal epithelial cell lineages other than goblet cells have been observed to take up luminal antigens.\textsuperscript{27,28} We observed that Paneth cells containing terminally administered Ova were present throughout the length of the SI but significantly less common when compared to goblet cells containing fluorescent Ova (Fig. 1a, b). We identified a small number of enteroendocrine cells containing luminally administered Ova in the steady state that were restricted to the duodenum; these were also significantly less common than goblet cells containing fluorescent Ova (Fig. 1a, b). In addition, we did not observe M cells in the non-follicle bearing epithelium in the SI or colon in the steady state (Fig. S1).

The currently favored route of luminal antigen acquisition by LP-APCs for tolerance induction is direct capture through the extension of TEDs. We evaluated the frequency and regional distribution of TED extension by LP-APCs by in vivo two-photon imaging of CD11c\textsuperscript{YFP} and CX\textsubscript{3}CR1\textsuperscript{GFP} reporter mice. Mice were imaged at various times throughout the day and were not deprived of food or water prior to imaging. At steady state conditions, we observed LP-APC extension of TEDs to be very rare in the distal SI and absent in the proximal SI (Fig. S1 B–E).

We observed two TEDs that were located in the distal SI out of greater than 500 villi imaged from tip to base from multiple CD11c\textsuperscript{YFP} reporter mice (Fig. S1D left side) and four TEDs forming in the distal SI out of greater than 350 villi imaged from tip to base throughout the SI from CX\textsubscript{3}CR1\textsuperscript{GFP/WT} reporter mice (Fig. S1E left side). We did not observe any TED extension in either the proximal or distal colon after analyzing 260 colonic crypts in the CD11c\textsuperscript{YFP} reporter mice and 263 crypts in the CX\textsubscript{3}CR1\textsuperscript{GFP} reporter mice (Fig. S1D and E, left side).

Previous studies removing the luminal contents mucus by washing, identified TED extension by APCs occurred at a rate of ~1.5–2.0 TEDs/villus.\textsuperscript{15–23} Approximately ten minutes following the removal of the luminal contents and mucus by rinsing with PBS, LP-APCs became less compact and extended multiple dendrites within the LP, into the epithelium, and into the lumen, with some LP-APCs traversing the epithelium (Supplemental Movie S1). However, consistent with prior observations,\textsuperscript{15,25} CX\textsubscript{3}CR1\textsuperscript{GFP+} LP-APC TED formation did not occur in the duodenum, the site where gavage antigen is acquired by CX\textsubscript{3}CR1\textsuperscript{GFP+} LP-APCs (Fig. S1E right side), and TED formation was not observed in the distal colon in any condition (Fig. S1B–E), consistent with observations by others that TEDs are rare or absent in the colon.\textsuperscript{31,32}

Using the in vivo two-photon imaging approach we used to evaluate the frequency of TEDs, we evaluated the frequency of GAPs in villous and colonic crypts. We did not observe an effect of removal of the mucus layer on the frequency of GAPs and the regional distribution of GAPs remained similar to our findings of GAPs using fluorescent microscopy on fixed tissue sections (data not shown). In the SI GAPs were ~1000 fold more common than TEDs when the mucus layer was left intact and ~10 fold more common when the mucus layer was removed (Fig. 1E). Thus, the frequency and regional distribution of GAPs, but not luminal antigen acquisition by Paneth cells, or enteroendocrine cells, the presence M cells, or LP-APC extension of TEDs correlated with regions of the gut where tolerance to luminal substances can be induced.\textsuperscript{13,14}

GAPs support LP-APC capture of, and CD4+ T cell responses to, luminal antigen

Mouse atonal homologue 1 (Math1) is a transcription factor required for the development of neurons and intestinal secretory intestinal epithelial lineages, which includes goblet cells, enteroendocrine cells, and Paneth cells.\textsuperscript{33–36} Paneth cells have a significantly longer half-life than goblet cells,\textsuperscript{37,38} and accordingly, 10 days after treatment with tamoxifen, mice with an inducible deletion of Math1 in intestinal epithelial cell lineages (Math1\textsuperscript{fl/fl}/vill-Cre-ER\textsuperscript{T2} mice) lose goblet cells (Fig. S2A), but retain Paneth cells, albeit at a somewhat reduced number when compared to their littermate controls (Fig. S2B). Goblet cells acquiring luminal fluorescent dextran in the SI and distal colon decreased significantly 10 days following the deletion of Math1 in intestinal epithelial cells (Fig. S2C and D). In contrast to the decrease in GAPs, intestinal permeability increased, as evidenced by serum levels of 4kD FITC dextran following gavage (Fig. 2a), and as evidence by the presence of 3kD fixable FITC dextran between epithelial cells and within the lamina propria of goblet cell deficient mice following gavage (Fig. S2E). The increased permeability might be attributed to the loss of the mucus barrier following goblet cell deletion. Despite the increase in intestinal permeability, SI LP-APCs, identified by flow cytometry (Fig. S3), acquired less luminally administered fluorescent Ova (Fig. 2b, c). In addition to the effects rising from the loss of GAPs, this may in part be related to the size of intact Ova (~43kD), as gavage of 40kD FITC dextran did not result in increased serum levels in goblet cell deficient mice (Fig. 2a) and gavaged fluorescent Ova was not found leaking between SI epithelial cells or in the lamina propria of goblet cell deficient mice (Fig. S2E). Isolation of the CD103+...
Goblet cell associated antigen passages support the induction and... DH, Kulkarni et al.

CD11b+ APC population and the CD103− CD11b+ APC population, which may contain DCs and macrophages, following Ova gavage revealed that the APCs were no longer inducing Ova specific CD4+ T cell responses in ex vivo co-cultures when goblet cells and GAPs were absent (Fig. 2d). We were unable to isolate sufficient numbers of CD103− CD11b+ SI LP-DCs for this ex vivo assay. The impaired ability of LP-APCs to induce T cell proliferation to luminal antigen was not due to an intrinsic defect in antigen acquisition or presentation, as LP-APCs isolated from mice with Math1 deleted in intestinal epithelial cells displayed no defects in capture of fluorescent antigen in culture (Fig. S4A) and no defect in induction of T cell proliferation when exogenous Ova was added to ex vivo co-cultures (Fig. S4B). We attributed the decrease in LP-APC antigen acquisition to the loss of goblet cells and GAPs, as we saw very few intestinal endoenteroendocrine cells and few Paneth cells acquiring luminal Ova in wild-type mice (Fig. 1a, b), and Paneth cells were still present at this time following deletion of Math1 (Fig. S2B). Moreover, mice lacking goblet cells/GAPs were significantly impaired at inducing antigen specific CD4+ T cell responses to gavaged antigen in the SI draining MLN (Fig. 2e), the site of tolerance induction to dietary antigens. The impaired responses to luminal antigen were not attributable to defects in the ability of MLN T cells to respond to Ova, as responses to systemically administered Ova were not impaired (Fig. S4C). The CSFE dilution seen in the mice lacking goblet cells may be due to antigen acquired at other sites, such as the Peyer’s Patches and migration of DCs to the MLN, as we saw no proliferation of OTII Rag−/− T cells, which have TCR specificity only for Ova, in the MLN in the absence of Ova gavage, and reduced but detectable proliferation of OTI Rag−/− T cells in the MLN in response to Ova in mice lacking goblet cells and GAPs when compared with their Cre− littermates (Fig. S4D). We also observed that GAPs were decreased in the distal colon of mice lacking goblet cells (Fig. S2D) and that LP-APC acquisition of intra-colonic fluorescent Ova was impaired in the distal colon in the absence of goblet cells and GAPs (Fig. 2f). Moreover, deletion of goblet cells impaired the induction of CD4+ T cell responses to Ova via enema in the distal colon draining LN in vivo (Fig. 2g). Thus, loss of goblet cells and GAPs impairs the ability of LP-APCs to acquire luminal antigen and impairs immune responses to luminal antigen in vivo despite the presence of increased intestinal leak.

Goblet cells play an important role in maintaining the intestinal barrier through mucus production and release of anti-microbial products, and accordingly deletion of goblet cells may have effects unrelated to the loss of GAPs. Therefore, to examine the role of the GAP function of goblet cells in luminal antigen delivery, we evaluated the effect of GAP inhibition on luminal antigen capture by LP-APCs and immune responses independent of deletion of goblet cells. GAPs form in response to acetylcholine (ACh) acting on the muscarinic ACh receptor 4 (mAChR4) on goblet cells, and Conversely GAPs are inhibited by activation of the epidermal growth factor receptor (EGFR) in goblet cells.21 Inhibition of GAPs by luminal recombinant murine epidermal growth factor factor (mEGF) significantly impaired LP-APC capture of luminally administered fluorescent Ova (Fig. 3a, b), as well as the ability of LP-APCs to acquire gavaged Ova in a manner capable of inducing antigen specific CD4+ T cell proliferation in ex vivo cultures (Fig. 3c). Moreover, mEGF significantly impaired antigen specific CD4+ T cell responses to oral Ova in vivo in the MLN (Fig. 3d). Importantly, deletion of the EGFR in goblet cells using an inducible Math1 driven Cre recombinase, EGFRf/fMath1CrePR mice, reversed the effects of mEGF on GAP inhibition, and T cell responses to luminal Ova in ex vivo cultures and in vivo (Fig. 3a, c, d), demonstrating that the defect in antigen capture could not be attributed to effects of EGFR on LP-APCs or T cells. This is consistent with the effect of EGF being mediated by effecting goblet cells and GAPs. Likewise, we observed that inducible deletion of mAChR4 on goblet cells, (mAChR4f/fMath1CrePR mice) did not affect goblet cell numbers (Fig. 3e), but impaired GAP...
formation (Fig. 3f). Unlike the deletion of goblet cells, we did not see an increase in leak when GAPs were inhibited (Fig. 3g) and accordingly we did not see a reduction in the mucus barrier when GAPs were inhibited and goblet cells remained intact (Fig. S5A and B). Inhibition of GAPs by deletion of the mAChR4 on goblet cells impaired luminal fluorescent Ova acquisition by LP-APCs (Fig. 3h, i), and impaired antigen specific CD4+ T cell responses to Ova in the SI draining MLN (Fig. 3j). We found that GAPs in the distal colon were inhibited by the pan-muscarinic acetylcholine receptor antagonist atropine and were induced by the ACh analogue carbamylcholine (Fig. S6A), but were not inhibited by deletion of mAChR4 in goblet cells (Fig. S6B), indicating that GAPs in the distal colon are induced by ACh acting on receptors other than mAChR4 and that there are yet to be identified pathways inducing GAP formation in the distal colon. While this prevented us from performing analogous studies in the distal colon to inhibit GAPs, these data support that the GAP function of goblet cells plays a role in delivering luminal antigens to LP-APCs for the induction of immune responses in the steady state.

Goblet cells and GAPs support the maintenance of Tregs and imprinting APCs in the LP

Tolerance to dietary antigens occurs in the SI and is mediated by CD4+ Foxp3+ pTregs that are generated in the draining LN. These pTregs subsequently traffic to and reside in the SI LP where they are maintained by continual stimulation by LP-APCs that have acquired the cognate antigen for these pTregs from the lumen.1,4,11,12 Accordingly, these pTregs may have a limited lifespan when their cognate antigen is withdrawn.13 In contrast, a substantial proportion of the pTregs residing in the colon LP differentiate in response to microbial stimuli and are longer-lived.13,39,40 A portion of these colonic pTregs can have specificity for gut bacterial antigens and their development requires GAPs in the proximal colon that are present for a defined period of time during a pre-weaning interval.41 This could suggest that luminal antigen delivery by GAP to LP-APCs might have a role in maintaining existing pTregs in the SI LP that have a more limited lifespan in the absence of continual stimulation. Indeed, we observed a decrease in the absolute number of SI LP Tregs when goblet cells/GAPs were deleted (Fig. 4a). This decrease largely affected the Helios- pTregs in the SI LP (Fig. 4b, c). We observed little change in the Helios- pTreg population in the colon LP (Fig. 4c). The relative lack of an effect of goblet cell/GAP deletion on the colonic pTreg population could reflect that adherent bacteria, which can induce immune responses by GAP independent endocytosis via enterocytes, can drive pTreg development in the colon in the steady state.42–44 Consistent with the pTregs being gut pTregs almost all of these LP Helios- Tregs
expressed the transcription factor RORγt (Fig. 4b), which can be expressed by SI LP pTregs with specificity to dietary antigens. Further, we observed that the SI LP pTreg population was reduced with GAP inhibition by mEGF in a goblet cell intrinsic EGFR dependent manner (Figs. 3a and 4d) and upon GAP inhibition by deletion of the mAChR4 in goblet cells (Figs. 3f and 4e), demonstrating that the GAP function of goblet cells facilitated the maintenance of SI LP pTregs.

Because the absence of GAPs impaired stimulation of Ova-specific T cells to dietary antigen in the MLN, and by extension would impair their differentiation to effector T cells or pTregs, we injected Ova intravenously to mice following adoptive transfer of OTII T cells to evaluate naive Ova-specific T cell differentiation in the absence of GAPs. We observed that in the absence of goblet cells, the in vivo induction of Tregs in the MLN in response to systemic Ova was impaired (Fig. 4f). The impaired ability to induce Tregs in response to dietary Ova in mice lacking goblet cells/GAPs can in part be attributed to defects in the population of LP-APCs isolated from mice lacking goblet cells as assessed by WGA staining, demonstrating that the GAP function of goblet cells facilitated the maintenance of SI LP pTregs.

SI LP-APCs consist of IRF8 dependent CD103+ CD11b− DCs, IRF4 dependent CD103+ CD11b+ DCs, and CD103− CD11b+ DCs and macrophages, which can express, but are not dependent upon IRF4.6,7 We observed a reduction in the CD103+ CD11b+ and CD103− CD11b+ populations, but not the CD103+ CD11b− DCs in the absence of goblet cells and GAPs (Fig. 5a). Accordingly, the absence of goblet cells and GAPs resulted in a decrease in the IRF4+ SI LP-APC population (Fig. 5b). The decrease in CD103+ CD11b+ and CD103− CD11b+ LP-APCs was dependent upon the GAP function of goblet cells as these populations were reduced in response to mEGF in an EGFR goblet cell dependent manner (Fig. 5c). CD103+ CD11b− and CD103+ CD11b+ SI LP-DCs can have aldehyde dehydrogenase activity,7 which facilitates the production of all-trans retinoic acid, a factor promoting the differentiation of and imprinting of Tregs with gut homing molecules.46 We observed that in the absence of goblet cells, SI LP-DCs had reduced aldehyde dehydrogenase (ALDH) activity (Fig. 5d) and an impaired ability to induce the expression of the gut homing molecules α4β7 and CCR9 on responding T cells in vitro co-cultures (Fig. 5e, f). Similar to the maintenance of pre-existing LP pTregs, SI LP-DC ALDH activity was facilitated by the GAP function of goblet cells, as this was impaired by GAP inhibition by mEGF in a goblet cell intrinsic EGFR dependent manner (Fig. 5g) and by GAP inhibition via the deletion of mAChR4 in goblet cells (Fig. 5h).

LP macrophages have been implicated in SI LP Treg maintenance through the production of IL-10 and stimulation of pre-existing pTregs with their cognate antigen acquired from the lumen.8 We found that mice lacking goblet cells and GAPs as well as mice with goblet cells but lacking GAPs had impaired IL-10 production by SI, but not colonic, CD11c+, MHCI+ CD11b+ F4/80+ LP-APCs (Fig. 5i, j), consistent with GAPs having a role in imprinting this LP-APC subtype in the SI. Thus, goblet cells and GAPs might contribute to multiple facets of oral tolerance including antigen delivery and imprinting LP-APCs for the induction and maintenance of pTregs specific for dietary antigens.

Goblet cells and GAPs support tolerance to dietary antigen

To directly evaluate the role for goblet cells and GAPs in tolerance to dietary antigens, mice lacking goblet cells and GAPs, their
Goblet cell associated antigen passages support the induction and... D.H. Kulkami et al.

Fig. 4  GAPs support the maintenance and induction of pTregs. a Absolute numbers of SI LP Tregs. b flow cytometry dot plots of Helios and RORγt expression by CD4+ Foxp3+ T cells in the SI LP and c quantification of ROηγt+ Helios- pTregs populations in the SI and colon LP of goblet cell deficient mice (Math1fl/fl vil-Cre-ERT2 mice) and littermate controls. Quantification of SI LP RORγt+ Helios- pTregs populations in d mice lacking EGFR in goblet cells (EGFRfl/fl Math1CrePR mice) and littermate controls treated with vehicle or mEGF, and in e mice lacking mAChR4 in goblet cells (mAChR4fl/fl Math1CrePR mice) and littermate controls. f Quantification of Foxp3 expression by MLN OTII T cells adoptively transferred into goblet cell deficient mice and littermate controls five days following i.v. injection of Ova. g Representative flow cytometry dot plots and quantification of Foxp3 expression by OTII T cells cultured for 5 days with Ova and SI LP-APCs isolated from goblet cell deficient mice and littermate controls. *P < 0.05, ns not significant. Data are presented as the mean ± SEM. Each data point represents an individual mouse.

littermate controls, and mice in which GAPs were transiently inhibited at the time of luminal antigen administration by intraluminal mEGF or deletion of mAChR4 in goblet cells, were gavaged with Ova, immunized with Ova, and evaluated for footpad swelling 24 h later. Goblet cell deficient mice and mice in which GAP formation was inhibited demonstrated significantly greater footpad swelling indicative of decreased tolerance to dietary antigen (Fig. 6a–d).

Moreover, deletion of EGFR in goblet cells at the time of mEGF and oral Ova administration reversed the effects mEGF on impaired tolerance, consistent with the effect of mEGF being due to GAP inhibition (Fig. 6c). Notably the Math1 Cre targets differentiated goblet cells, which turn over every 3–5 days, and therefore the inhibition of GAPs by deletion of mAChR4 and the reversal of effects of EGFR by deletion of EGFR in goblet cells is largely limited to the time of luminal Ova administration, and not due to effects on goblet cells at the time of Ova immunization and challenge. While the mAChR4 independent formation of GAPs in the distal colon prevented us from directly assessing the role of the GAP function of goblet cells in tolerance to luminal antigens in the colon, we did observe that deletion of goblet cells impaired the ability to induce tolerance to Ova administered via enema (Fig. 6e). Loss of goblet cells might induce inflammatory responses due to the deficient mucus barrier, which could affect the capture of luminal substances by resident LP-APCs and the induction of tolerance independent of the loss of GAPs. Indeed, we observed that deletion of goblet cells resulted in an increase in monocytes and neutrophils in the SI lamina propria (Fig. 5A). However, we did not see an increase in monocytes in the lamina propria when GAPs were inhibited and goblet cells remained intact (Fig. 5B) suggesting that inflammatory responses alone do not account for the loss of tolerance when GAPs are inhibited. Mice with goblet cell and GAP manipulation had increased serum levels of interferon-γ (IFNγ) following immunization (Fig. 6f–i), correlating with their loss of tolerance to dietary Ova. The impaired tolerance in the absence of goblet cells or GAPs was not as severe as that seen in the absence of luminal Ova exposure (Fig. 6a–e), suggesting the potential for other or compensatory routes of luminal antigen delivery in the absence of goblet cells and GAPs. However, in total these observations indicate that GAPs support the induction of tolerance to luminal antigens on multiple levels.

DISCUSSION

The gut lumen contains trillions of microbes and abundant microbial products. Inducing and maintaining tolerance to innocuous substances originating from this potentially inhospitable environment is fundamental to maintaining homeostasis and health. Indeed, tolerance is so effectively induced to antigens originating from the gut lumen that oral tolerance regimens are being leveraged to treat extra-intestinal diseases. Accordingly, how tolerance is induced and maintained at this mucosal surface has been a topic of many studies.

The gut microenvironment has unique properties supporting tolerance. Tolerance to non-self antigens is largely mediated by the conversion of naïve T cells into Foxp3 expressing pTregs, which is facilitated by a local environment containing all-trans retinoic acid (ATRA) and TGFβ. ATRA, are sources of ATRA supporting pTreg induction and imprinting gut homing molecules on lymphocytes. DC imprinting with retinaldehyde dehydrogenase activity is induced by luminal retinoids and by DC association with the intestinal epithelium. Moreover, the goblet cell protein, mucin 2, promotes tolerogenic properties in DCs inducing pTregs including the production of TGFβ and the expression of retinaldehyde dehydrogenase, and select members of the gut microbiota promote pTregs through bacterial products or metabolites.

Thus, these unique properties contribute to the tolerogenic tone of the gut environment, yet how luminal antigens are acquired by the immune system for the induction of tolerance and whether this process contributes to tolerance beyond antigen capture have been unexplored. How luminal antigens are encountered by the immune system may affect the phenotype of the subsequent immune response.
A landmark discovery identified that LP-APCs had the ability to extend dendrites between epithelial cells to capture luminal bacteria without compromising the epithelial barrier, suggesting that this process might allow minimally disruptive direct capture of luminal substances. However, LP-APC extension of TEDs is absent in some mouse strains, suggesting that unlike oral tolerance, LP-APC TED extension is not a universal phenomenon and other pathways of luminal antigen capture inducing oral tolerance exist. In addition, while the extension of TEDs is impaired in the absence of CX3CR1, CD4+ T cell responses to luminal antigens are not, suggesting that the defect in oral tolerance in CX3CR1-deficient mice was unrelated to luminal antigen capture. We observed that the extension of TEDs by LP-APCs is very rare in the steady state but became more common after the removal of the luminal contents and mucus layer, occurring in a frequency similar to prior reports. Why removal of the luminal contents and mucus layer induces TED extension is unclear, but could be related to the release of lactate and pyruvate by stressed epithelial...
cells as these metabolites were recently identified to induce TED extension in CX3CR1+ LP-APCs, and we have observed that TED extension occurs when mice expire while imaging under anesthesia in the absence of removal of the luminal contents and mucus layer (unpublished observation). We did not observe LP-APC TED extension in the duodenum, the site where gavaged antigen is acquired by CX3CR1+ LP-APCs, or in the distal colon, the site where tolerance to luminal antigen is induced in the colon. While it is impossible to exclude a contribution of LP-APC TED extension, combined with the above observations, these findings indicate that LP-APC TED extension is less likely to be a major route of steady state soluble luminal antigen capture for the induction of oral tolerance.

Early observations suggested that M cells were restricted to the epithelium overlying the Peyers' patches; however, subsequent studies identified M cells overlying the non-follicle bearing villous epithelium. Villous M cells are rare in the steady state but can be induced by systemic treatment with TNF superfamily member receptor activator of NF-κB ligand (RANKL), whose expression is normally restricted to subepithelial stromal cells restricted to the Peyers' patches. These villous M cells can be closely associated with goblet cells and have the capacity to transcytose bacteria to induce immune responses to luminal bacteria. We also found villous M cells to be very rare in the steady state, suggesting they are not a major pathway for luminal antigens to traverse the epithelium to support oral tolerance. Similar barrier leak, as evidenced by the presence of luminally administered 4 kD dextran in the serum did not correlate with LP-APCs acquisition of luminal antigen. Why barrier leak is less effective at loading LP-APCs with antigen in a manner capable of inducing T cell responses is unclear but could be related to the size of substances delivered via paracellular leak relative to the size of proteins/polypeptides required to induce antigen specific T cell responses as we did not see an increase in 40 kD dextran in the serum following gavage in goblet cell deficient mice.

In contrast, the presence of intestinal epithelial cells filling with luminal antigen was common in the steady state. Consistent with a recent report, we observed enteroendocrine cells containing luminal antigen; however, they were rare and limited to the duodenum in the steady state. We more commonly observed Paneth cells containing luminal antigen, but these were still relatively rare occurring on average in one Paneth cell in every two crypt cross sections. Moreover, Paneth cells are less likely to be a major contributor to steady state luminal antigen delivery supporting tolerance as they are absent from the colon, and due to their longer lifespan, persist for weeks following deletion of Math1 in epithelial cells, which we observed results in significantly impaired luminal antigen delivery to LP-APCs. In contrast, goblet cells filling with luminal antigen were commonly observed in the regions of the gut where luminal antigens are acquired to induce tolerance, suggesting that goblet cells and GAPs may be pathways delivering dietary antigens to LP-APCs to support oral tolerance.

Paneth cells containing luminal antigen, but these were still relatively rare occurring on average in one Paneth cell in every two crypt cross sections. Moreover, Paneth cells are less likely to be a major contributor to steady state luminal antigen delivery supporting tolerance as they are absent from the colon, and due to their longer lifespan, persist for weeks following deletion of Math1 in epithelial cells, which we observed results in significantly impaired luminal antigen delivery to LP-APCs. In contrast, goblet cells filling with luminal antigen were commonly observed in the regions of the gut where luminal antigens are acquired to induce tolerance, suggesting that goblet cells and GAPs may be pathways delivering dietary antigens to LP-APCs to support oral tolerance. Of note GAPs are present in strains of mice in which the extension of TEDs by LP-APCs is absent. Indeed, we observed that in the absence of goblet cells and GAPs luminal antigen capture by CD103−CD11b+ LP-APCs was impaired. Our initial observation of GAP mediated antigen delivery to LP-APCs reported that GAPs delivered antigen to CD103−LP-DCs. Other APC populations could acquire luminal antigen and stimulate antigen specific T cell responses when GAPs were induced above baseline levels, suggesting that GAPs deliver antigen to these APC populations as well. We have observed CD103−LP-APC populations interacting with GAPs in the SI and colon. The preferential ability of CD103+ LP-APCs over CD103−LP-APCs to induce T cell responses to luminal antigen may be related to their enhanced antigen presentation and stimulation capacities or may be due to passage of antigen from CD103−LP-APCs to CD103+LP-DCs. Irrespective of the pathway by which CD103+LP-DCs acquire luminal antigen, either by direct capture from goblet cells, or from transfer from CD103−LP-APCs, our observations indicate that this process is supported by the GAP function of goblet cells. While luminal antigen capture by
LP-APCs was nearly undetectable in the absence of GAPs, proliferation of dietary antigen specific T cells in the MLN and oral tolerance, as measured by DTH responses, were less dramatically impaired. This could be consistent with contributions of dietary antigen capture at other sites, such as the Peyer’s patches, to T cell responses in the MLN and tolerance to dietary antigens.

The findings presented here indicate that GAPs function beyond simple antigen delivery to promote oral tolerance. LP-DCs with ALDH activity produce all-trans retinoic acid, which promotes the induction of pTregs. Further, the production of IL-10 by resident LP macrophages supports the expansion and maintenance of pre-existing LP pTregs specific for dietary antigens. We found that in the absence of goblet cells/GAPs imprinting SI LP-APCs with ALDH activity and the production of IL-10 by SI macrophages were impaired. When CD103+ LP-DCs acquire luminal antigens from GAPs, they also acquire goblet cell proteins. Combined with observations that the goblet cell protein mucin 2 imprints DCs with antigens away from tolerance toward Th17 responses.81 In the context of the findings presented here, this suggests that CD103+ LP-DC imprinting by GAPs may occur during antigen acquisition and that GAPs may deliver tolerogenic signals in concert with luminal substances to support antigen specific tolerance induction. The mechanism of tolerance induction to luminal substance encountered in the distal colon differs from that of the SI and can utilize other APC populations.14 While we did not observe LP-APCs defects in the colon in the absence of goblet cells/GAPs, the relevant properties of the APCs inducing tolerance in the distal colon are not known, and accordingly whether GAPs in the distal colon play an analogous role influencing this APC phenotype remains to be investigated. Beyond this we noted that GAPs supported the maintenance of pre-existing pTregs in the SI LP. These pTregs reexpressed within days of GAP inhibition, a time course that is much faster than regression of SI LP pTregs when deprived of cognate antigen.13 This suggests that GAPs may play additional yet to be identified roles beyond antigen delivery in shaping the immune landscape of the gut. Related to this, enteric viral infection abrogates oral tolerance and promotes Th1 immune responses to dietary antigen, and enteric bacterial infection inhibits GAPs and shifts immune responses to dietary antigen away from tolerance toward Th17 responses.81 In the context of the findings presented here, this might suggest that GAP inhibition during enteric infection is a physiologic response facilitating inflammatory responses for pathogen clearance.

Immune tolerance to innocuous substances encountered in the gut lumen is a recognized phenomenon that is essential for gut health. How this process occurs is a fundamental question. Here we identify a role for goblet cells and GAPs as routes for luminal antigen encounter by the immune system for the induction of tolerance to dietary antigens in the steady state. Moreover, we observed that GAPs imprint LP-APCs with properties necessary for the induction and maintenance of pTregs. Combined with studies demonstrating that GAP formation is closely regulated to prevent inappropriate inflammatory responses to luminal substances encountered in hostile settings, and that GAPs promote the induction of antigen specific tolerance to commensal bacteria during a defined pre-weaning interval, the observations presented here suggest that goblet cell and GAP dysfunction may contribute to the pathogenesis of intestinal inflammatory diseases. Moreover, these observations suggest that restoring goblet cell and GAP function may be one component of approaches to restore gut immune homeostasis.

METHODS
See online supplementary information for complete methods.

Mice
All mice were 10 or more generations on the C57BL/6 background, with the exception of the mACHR4fl/fl mice, which were 6–7 generations on the C57BL/6 background at the time of these studies. C57BL/6 mice, congenic CD45.1 B6SJL mice, OTII T cell receptor transgenic mice, CD11ccre transgenic mice, CX3CR1GFP mice, Math1cre mice, FoxP3GFP mice, were purchased from The Jackson Laboratory (Bar Harbor, ME) or The National Cancer Institute (Frederick, MD). Transgenic mice in which a tamoxifen-dependent Cre recombinase is expressed under the control of the villin promoter (vil-CreERT2) were a gift from Sylvie Robine (Institut Curie, Paris, France). Math1cre mice were bred to vil-CreERT2 mice to generate mice with inducible deletion of goblet cells following deletion of Math1 in villin expressing cells. Math1cre/villin-CreERT2 mice and the injection protocol to induce goblet cell deletion have been previously described. EGFRfr mice were a gift from Dr. David Threadgill, University of North Carolina. mACHR4cre mice were a kind gift from Jurgen Wess (National Institute Health, Bethesda, MD). EGFRfr mice and mACHR4cre mice were bred to Math1cre mice to generate mice with an inducible deletion of EGFR or mACHR4 in goblet cells. Mice were housed in a specific-pathogen-free facility and fed routine chow diet. Mice of both sexes were used in this study. Animal procedures and protocols were performed in accordance with the IACUC at Washington University School of Medicine.

Intravitral two-photon (2 P) microscopy
In vivo two-photon imaging was performed as previously described.20 Evaluation of luminal antigen uptake by epithelial cells
Tetramethylrhodamine-labeled 10 kD dextran or Texas Red labeled ovalbumin was administered in the SI, proximal and distal colon of anesthetized mice. After 1 h, mice were sacrificed, and tissues thoroughly washed with cold PBS before fixing in 10% formalin buffer solution. Tissues were embedded in optimal cutting temperature compound (Fisher Scientific, Pittsburgh, PA) and 6 μm sections prepared. For studies in Fig. 1, sections were stained with wheat germ agglutinin (WGA), Ulex europaeus agglutinin I (UEA I), anti-lysozyme antibodies, or anti-chromogranin A antibodies to identify goblet cells in the SI, goblet cells in the colon, Paneth cells, and enteroendocrine cells respectively. Sections were then stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, St Louis, MO) and imaged using an Axioskop 2 microscope with a Plan-Neofluar ×20/0.5 objective (Carl Zeiss Microscopy, Thornwood, NY).

Analysis of luminal fluorescent antigen uptake by LP-APCs
Mice were anesthetized and 200 μg of Alexa Fluor 647 labeled ovalbumin (Ova-A647), dissolved in phosphate buffered saline (PBS), or PBS alone (controls), was injected into the SI lumen, or given via enema using a 16 G plastic cannula inserted 3 cm transanal into the colon. In some experiments, anesthetized mice were treated intraluminally with 10 μg murine EGF (Shenandoah Biotechnology, Warwick, PA) dissolved in PBS, or PBS alone 20 min prior to Ova-A647 administration. Two hours later cellular populations were isolated from the non-Peyer’s patch bearing SI or distal colon as described previously.85 The distal colon segment represents the last two cm of the colon. Isolated LP cells were stained for APC markers and evaluated for Ova-A647 positive staining by flow cytometry.

Analysis of luminal antigen delivery to LP-APCs and induction of T cell proliferation in vitro
Mice were anesthetized and 2 mg of ovalbumin (Ova) dissolved in phosphate buffered saline (PBS), or PBS alone (controls), was injected intraluminally into the SI. For delivery of Ova by enema or a 16 g plastic cannula was inserted 3 cm transanal into the colon. In some experiments, anesthetized mice were intraluminally treated with 10 μg murine EGF (Shenandoah Biotechnology,
Warwick, PA) 20 min prior to Ova administration. Two hours later cellular populations were isolated from the non-Peyer’s patch bearing SI LP. APC populations and Ova-specific CD4+ OTII T cells were isolated with flow-cytometric cell sorting and cultured at a ratio of 1:10 APCs (1 × 10^4) to T cells (1 × 10^5). As a positive control, 10 µg Ova was added to cultures of APC populations isolated from mice receiving luminal PBS. After 3 days, cultures were evaluated for the number of T cells by flow cytometry and cell counting.

Adaptive T cell transfer and analysis of in vivo antigen specific T cell responses to luminal Ova
To evaluate the role of goblet cells and GAPs on delivery of luminal antigen and antigen specific T cell proliferation in the draining lymph nodes, single-cell suspensions of Ova-specific T cells were prepared from spleens and MLNs of CD45.1+ OTII T cell receptor transgenic mice, and CD4 T cell enrichment was performed using magnetic beads (Stemcell Technology, Vancouver, BC). Enriched CD4+ T cells were labeled with 2 µM CFSE (Invitrogen, Carlsbad, CA) and 2 × 10^5 CFSE-labeled cells were i.v. transferred into sex matched recipient mice. Twenty-four hours after transfer, mice were orally gavaged with 15 mg Ova (Sigma–Aldrich, St. Louis, MO) or in some experiments mice were administered 25 mg of ovalbumin via enema using a 16 G plastic cannula as above. EGRFfl/ or EGRFfl/Math1cre mice were, administered with 10 µg of murine EGF 20 min prior to receiving 15 mg ovalbumin in saline orally. Two days later SI draining MLNs or distal colon draining caudal and iliac LN were removed and single-cell suspensions were prepared and analyzed by flow cytometry for CD45.1, CD3, Vβ5, Vα2, and CFSE. To evaluate the effect of systemic antigen administration on transferred T cells, 24 h post adoptive transfer 200 µg of Ova was administered i.v. and transferred T cells evaluated on the same schedule as described above.

pTreg generation in vivo and in vitro
To evaluate de novo induction of pTreg cells, single-cell suspensions from spleen and MLNs from Ova-specific CD45.1+ Foxp3GFP OTII T cell receptor transgenic mice were flow cytometrically sorted for GFP+, Vβ5+, Vα2+, CD45.1+, CD62hi cells. 5 × 10^5 cells were i.v. administered into recipient Math1cre/vil-Cre-ERT2 or Math1cre mice 7 days after start of tamoxifen treatment. Recipient mice were gavaged with 15 mg Ova, and SI draining MLNs were evaluated five days later for Foxp3GFP+ cells among the transferred cells. To evaluate the de novo generation of pTregs in vitro naive Foxp3GFP+ CD45.1+ OTII T cells were isolated as above and cultured with flow cytometrically sorted LP-APCs at a ratio of 10:1 with 40 µg of exogenous Ova. Five days later cultures were harvested and evaluated for Foxp3GFP+ expression by T cells.

ALDH activity
To evaluate the expression of ALDH in DCs, intestinal LP cells were stained using ALDEFLUOR (StemCell Technologies, Vancouver, BC, Canada) per the manufacturer’s recommendations as previously described.35

Analysis of CCR9 and α4β7 induction by T cell in vitro
Cellular populations were isolated from the non-Peyer’s patch bearing SI LP of mice lacking goblet cells and littermate controls. CD11c+ MHCIi+ CD103+ CD11b+ populations and Ova-specific CD4+ OTII T cells were isolated with flow-cytometric cell sorting. Cell were cultured at a ratio of 1:10 APCs (1 × 10^4) to T cells (1 × 10^5) and 2 µg Ova was added each well. After 3 days, cultures were evaluated for the expression of CCR9 and α4β7 on T cells by flow cytometry.

Measurement of mucus thickness
To determine the thickness of mucus layer, SI tissue containing luminal matter were fixed in Carnoy’s fixative overnight. Subsequently, tissues were passed reducing concentration of methanol, before being embedded in OCT. Tissue sections were cut to a thickness of 6 µm and slides were dried to room temperature before staining with Alcian Blue for mucus.

Oral tolerance and delayed type hypersensitivity responses
Mice were given Ova 20 g/L in drinking water, or drinking water alone for 2 weeks, or alternatively were gavaged with 20 mg Ova daily for 2 weeks concurrent with gavage of 10 µg murine EGF or given 25 mg Ova via enema. Two weeks and four weeks following dietary Ova exposure mice were immunized subcutaneously with 100 µg Ova in incomplete Freund’s Adjuvant (Sigma–Aldrich). Two weeks after the last immunization mice were challenged with 20 µg Ova in the footpad and the change in footpad thickness evaluated using measurements taken with micrometer calipers before and 24 h after challenge. Blood was collected 24 h after footpad challenge and serum levels of IFNγ were measured using Mouse IFNy ELISA kit (eBioscience, San Diego, CA), according to manufacturer’s protocol.

Statistical analysis
Data analysis using a two sided student’s t-test for studies involving two groups or one way ANOVA with a Dunnett’s or Tukey’s post-test with correction for multiple comparisons for studies involving three or more groups was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). A cut-off of p < 0.05 was used for significance.

ACKNOWLEDGEMENTS
Supported by grants: DK097317, A131342, A112626, DK109006, A1136515, AI 140755, and Crohn’s and Colitis Foundation Research Fellowship Award 348359 and Swedish Research Council International Postdoc Award 2014-00366. The authors wish to thank Mark J Miller for advice and assistance with in vivo two-photon imaging. The Washington University Digestive Diseases Research Center Core, supported by NIH grant P30 DK052574 assisted with imaging. Two photon in vivo imaging was performed at the Washington University School of Medicine In Vivo Imaging Core. The High Speed Cell Sorter Core at the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, MO. provided flow-cytometric cell sorting services. The Siteman Cancer Center is supported in part by NCI Cancer Center Support Grant P30 CA91842.

AUTHOR CONTRIBUTIONS
D.H.K., J.K.G., K.A.K., K.G.M., A.N.F., S.S.B., and J.E.D. performed the experiments. D.H.K., J.K.G., S.P.H., C.S.H., and R.D.N. designed the study. D.H.K., J.K.G., S.P.H., C.S.H., and R.D.N. wrote the manuscript. All authors have reviewed and agree with the manuscript content.

ADDITIONAL INFORMATION
The online version of this article (https://doi.org/10.1038/s41385-019-0240-7) contains supplementary material, which is available to authorized users.

Competing interests: R.D.N., K.A.K., and K.G.M. are inventors on U.S. Nonprovisional Application Serial No. 15/880,658 Compositions And Methods For Modulation Of Dietary And Microbial Exposure.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES
1. Pabst, O. & Mowat, A. M. Oral tolerance to food protein. Mucosal Immunol. 5, 232–239 (2012).
2. Bogunovic, M. et al. Origin of the lamina propria dendritic cell network. Immunity 31, 513–525 (2009).
3. Varol, C. et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity 31, 502–512 (2009).
4. Schulz, O. et al. Intestinal CD103−, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J. Exp. Med. 206, 3101–3114 (2009).
5. Persson, E. K. et al. IRF4 transcription-factor-dependent CD103([+)CD11b([-) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* **38**, 958–969 (2013).

6. Schützer, A. et al. IRF4 transcription factor-dependent CD11b([+) dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* **38**, 970–983 (2013).

7. Lydia, K. M. et al. IRF8 transcription-factor-dependent classical dendritic cells are essential for intestinal T cell homeostasis. *Immunity* **44**, 860–874 (2016).

8. Esterhazy, D. et al. Classical dendritic cells are required for dietary antigen-mediated induction of peripheral Treg cells and tolerance. *Nat. Immunol.* **17**, 545–555 (2016).

9. Spahn, T. W. et al. Mesentric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer’s patches. *Eur. J. Immunol.* **32**, 1109–1113 (2002).

10. Spahn, T. W. et al. Induction of oral tolerance to cellular immune responses in the absence of Peyer’s patches. *Eur. J. Immunol.* **31**, 1278–1287 (2001).

11. Hadis, U. et al. Intestinal tolerance requires gut homing and expansion of FoxP3([+) T regulatory cells in the lamina propria. *Nature* **437**, 24–26 (2005).

12. McDole, J. R. et al. Goblet cells deliver luminal antigen to CD103([-) dendritic cells in the murine small intestine. *Dev. Dyn.* **233**, 1332–1336 (2005).

13. Troughton, W. D. & Trier, J. S. Paneth and goblet cell renewal in mouse duodenal crypts. *J. Cell Biol.* **41**, 251–268 (1969).

14. Knoop, K. A. et al. Individual intestinal symbions stimulate a distinct population of RORgamma T regulatory cells. *Science* **349**, 993–997 (2015).

15. Knoop, K. A. et al. Microbial antigen encounter during a preweaning interval is critical for tolerance to gut bacteria. *Sci. Immunol.* [https://doi.org/10.1126/sciimmunol.aaq2633](https://doi.org/10.1126/sciimmunol.aaq2633) (2015).

16. Rescigno, M. et al. Dendritic cells express tight junction proteins and penetrate the gut epithelial monolayers to sample bacteria. *Proc. Natl Acad. Sci. USA* **99**, 595 (2002).

17. Esterhazy, D. et al. Classical dendritic cells are required for dietary antigen-mediated induction of peripheral T regulatory cells and tolerance. *Nat. Immunol.* **14**, 595 (2013).

18. Jang, M. H. et al. Intestinal villous M cells: an antigen entry site in the mucosal immune system and relies on antigen carriage by dendritic cells. *J. Exp. Med.* **203**, 519–527 (2006).

19. Kim, K. S. et al. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science* [https://doi.org/10.1126/science.aac5560](https://doi.org/10.1126/science.aac5560) (2016).

20. Veenbergen, S. et al. Colonic tolerance develops in the iliac lymph nodes and can be established independent of CD103 dendritic cells. *Mucosal Immunol.* [https://doi.org/10.1038/mi.2015.118](https://doi.org/10.1038/mi.2015.118) (2015).

21. Chieppa, M., Rescigno, M., Huang, A. Y. & Germain, R. N. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J. Exp. Med.* **203**, 2841–2852 (2006).

22. Rescigno, M. et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2**, 361–367 (2001).

23. Shen, L., Weber, C. R., Rleigh, D. R., Yu, D. & Turner, J. R. Tight junction pore and leak pathways: a dynamic duo. *Annu. Rev. Physiol.* **73**, 283–309 (2011).

24. Jang, M. H. et al. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc. Natl Acad. Sci. USA* **101**, 6110–6115 (2004).

25. Terahara, K. et al. Comprehensive gene expression profiling of Peyer’s patch M cells, villous M-like cells, and intestinal epithelial cells. *ImmunoL. (Baltimore, Md.)*** **180**, 7840–7846 (2008).

26. McDole, J. R. et al. Goblet cells deliver luminal antigen to CD103([-) dendritic cells in the small intestine. *Nature* **483**, 345–349 (2012).

27. Knoop, K. A., McDonald, K. G., McCrate, S., McDole, J. R. & Newberry, R. D. Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol.* **8**, 198–210 (2015).

28. Kulkarni, D. H. & Newberry, R. D. Intestinal Macromolecular Transport Supporting Adaptive Immunity. *Cell. Mol. Gastroenterol. hepatol.* **7**, 729–737 (2019). [https://doi.org/10.1016/j.jcmgh.](https://doi.org/10.1016/j.jcmgh.)

29. Rimoldi, M. et al. Monocyte-derived dendritic cells activated by bacteria or by bacteria-stimulated epithelial cells are functionally different. *Blood* **106**, 2818–2826 (2005).

30. Vallon-Eberhard, A., Landsman, L., Yogev, N., Verrier, B. & Jung, S. Transepithelial pathogen uptake into the small intestinal lamina propria. *ImmunoL. (Baltimore, Md.)*** **170**, 2465–2469 (2006).

31. Niess, J. H. et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* **307**, 254–258 (2005).

32. Mazzini, E., Massimiliano, L., Penna, G. & Rescigno, M. Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1([+) macrophages to CD103([-) dendritic cells. *Immunity* **40**, 248–261 (2014).

33. Nagatake, T., Fujita, H., Ninato, N. & Hamazaki, Y. Enteroidinococytes are specifically marked by cell surface expression of claudin-4 in mouse small intestine. *PLoS One* **9**, e90638 (2014).

34. Noah, T. K. et al. IL-3-induced Intestinal secretory epithelial cell antigen passages are required for IgG-mediated food-induced anaphylaxis. *J. Allergy Clin. Immunol.* [https://doi.org/10.1016/j.jaci.2019.04.030](https://doi.org/10.1016/j.jaci.2019.04.030) (2019).

35. Farach-Marquez, J. et al. Intestinal bacteria recruit CD103([-) dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity* **38**, 581–595 (2013).

36. Kim, K. W. et al. In vivo structure/function and expression analysis of the CX3C chemokine fractalkine. *Blood* **118**, e156–e167 (2011).

37. Hafemseier, S. et al. Microbe sampling by mucosal dendritic cells is a discrete, MyD88-independent step in DeltavinS S. Typhimurium colitis. *J. Exp. Med.* **205**, 437–450 (2008).

38. Cruickshank, S. M. et al. Rapid dendritic cell mobilization to the large intestinal epithelium is associated with resistance to Trichuris muris infection. *J. Immunol.* **182**, 3055–3062 (2009).

39. Shroyer, N. F. et al. Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. *Gastroenterology* **132**, 2478–2488 (2007).
Goblet cell associated antigen passages support the induction and...  
DH. Kulkarni et al.

63. Shan, M. et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science* **342**, 447–453 (2013).
64. Atarashi, K. et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* **331**, 337–341 (2011).
65. Atarashi, K. et al. T induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature*, https://doi.org/10.1038/nature12331 (2013).
66. Round, J. L. & Mazmanian, S. K. Inducible Foxp3⁺ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc. Natl Acad. Sci. USA* **107**, 12204–12209 (2010).
67. Mazmanian, S. K., Round, J. L. & Kasper, D. L. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* **453**, 620–625 (2008).
68. Chu, H. et al. Gene-microbiota interactions contribute to the pathogenesis of inflammatory bowel disease. *Science* **352**, 1116–1120 (2016).
69. Furusawa, Y. et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* **504**, 446–450 (2013).
70. Arpaia, N. et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**, 451–455 (2013).
71. Foti, M. & Ricciardi-Castagnoli, P. Antigen sampling by mucosal dendritic cells. *Trends Mol. Med.* **11**, 394–396 (2005).
72. Schulz, O. & Pabst, O. Antigen sampling in the small intestine. *Trends Immunol.* **34**, 155–161 (2013).
73. Knoop, K. A., Miller, M. J. & Newberry, R. D. Transepithelial antigen delivery in the small intestine: different paths, different outcomes. *Curr. Opin. Gastroenterol.* **29**, 112–118 (2013).
74. Rescigno, M., Rotta, G., Valzasina, B. & Ricciardi-Castagnoli, P. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* **204**, 572–581 (2001).
75. Morita, N. et al. GPR31-dependent dendrite protrusion of intestinal CX3CR1(+) cells by bacterial metabolites. *Nature*, https://doi.org/10.1038/s41586-019-0884-1 (2019).
76. Knoop, K. A. et al. RANKL is necessary and sufficient to initiate development of antigen-sampling M cells in the intestinal epithelium. *J. Immunol.* **183**, 5738–5747 (2009).
77. Hase, K. et al. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. *Nature* **462**, 226–230 (2009).
78. Knoop, K. A., McDonald, K. G., Kulkarni, D. H. & Newberry, R. D. Antibiotics promote inflammation through the translocation of native commensal colonic bacteria. *Gut* **65**, 1100–1106 (2016).
79. Knoop, K. A. et al. Antibiotics promote the sampling of luminal antigens and bacteria via colonic goblet cell associated antigen passages. *Gut Microbes* https://doi.org/10.1080/19490076.2017.1299846 (2017).
80. Bouzait, R. et al. Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. *Science* **356**, 44–50 (2017).
81. Kulkarni, D. H. et al. Goblet cell associated antigen passages are inhibited during Salmonella typhimurium infection to prevent pathogen dissemination and limit responses to dietary antigens. *Mucosal Immunol.* https://doi.org/10.1038/s41385-018-0007-6 (2018).
82. Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. Defective TCR expression in bacteria via colonic goblet cell associated antigen passages. *Mucosal Immunol.* https://doi.org/10.1038/nature12331 (2013).
83. Lindquist, R. L. et al. Visualizing dendritic cell networks in vivo. *Nat. Immunol.* **5**, 1243–1250 (2004).
84. Jung, S. et al. Analysis of fractalkine receptor CX3CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell Biol.* **20**, 4106–4114 (2000).
85. Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. Regulatory T cells prevent catastrophic autoimmune throughout the lifespan of mice. *Nat. Immunol.* **8**, 191–197 (2007).
86. el Marjou, F. et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genes* **39**, 186–193 (2004).
87. Lee, T. C. & Threadgill, D. W. Generation and validation of mice carrying a conditional allele of the epidermal growth factor receptor. *Genes* **47**, 85–92 (2009).
88. Jeon, J. et al. A subpopulation of neuronal M4 muscarinic acetylcholine receptors plays a critical role in modulating dopamine-dependent behaviors. *J. Neurosci.* **30**, 2396–2405 (2010).