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Goblet Cell Associated Antigen Passages Support the Induction and Maintenance of Oral Tolerance

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Abstract

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.

Keywords

Oral Tolerance; Intestine; Goblet Cell; T Regulatory Cell; Dietary Antigens

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Author contributions

DHK, JKG, KAK, KGM, ANF, SSB, and JED performed the experiments. DHK, JKG, KGM, SPH, CSH, and RDN designed the study. DHK, JKG, SPH, CSH, and RDN wrote the manuscript. All authors have reviewed and agree with the manuscript content.

Declaration of Interests

RDN, KAK, and KGM are inventors on U.S. Nonprovisional Application Serial No. 15/880,658 Compositions And Methods For Modulation Of Dietary And Microbial Exposure.

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 underlying 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 extension 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 [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 find that the GAP function of goblet cells imprints and maintains LP-DCs and macrophages with tolerogenic properties, maintains pre-existing Tregs in the SI LP, and in the absence of GAP tolerance to 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

In the steady state, tolerance to luminal substances is induced in the SI and distal colon [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. Immunofluorescent staining of fixed tissue sections demonstrated that goblet cells containing the luminally 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 (Figure 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 [13,14]. Secretory intestinal epithelial cell lineages other than goblet cells have been observed to take up luminal antigens [27,28]. We observed that Paneth cells containing luminally administered Ova were present throughout the length of the SI but significantly less common when compared to goblet cells containing fluorescent Ova (Figure 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 (Figure 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 (Figure 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 $CD11c^{YFP}$ and CX_3CR1^{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 (Figure 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 $CD11c^{YFP}$ reporter mice (Figure 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 $CX_3CR1^{GFP/WT}$ reporter mice (Figure S1E left side). We did not observe any TED extension in either the proximal or distal colon after analyzing 260 colonic crypts in the $CD11c^{YFP}$ reporter mice and 263 crypts in the CX_3CR1^{GFP} reporter mice (Figure 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 [15,24,25,29,30]. 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 [15,25], CX_3CR1^{GFP+} LP-APC TED formation did not occur in the duodenum, the site where gavage antigen is acquired by CX_3CR1^{GFP+} LP-APCs (Figure S1E right side), and TED formation was not observed in the distal colon in any condition (Figure S1B–E), consistent with observations by others that TEDs are rare or absent in the colon [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 villi 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 (Figure 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 [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 [33–36]. Paneth cells have a significantly longer half-life than goblet cells [37,38], and accordingly, ten days after treatment with tamoxifen, mice with an inducible deletion of Math1 in intestinal epithelial cell lineages ($Math1^{fl/fl}$vil-Cre-ER^{T2} mice) lose goblet cells (Figure S2A), but retain Paneth cells, albeit at a somewhat reduced number when compared to their littermate controls (Figure S2B). Goblet cells acquiring luminal fluorescent dextran in the SI and distal colon decreased significantly ten

days following the deletion of Math1 in intestinal epithelial cells (Figure S2C and D). In contrast to the decrease in GAPs, intestinal permeability increased, as evidenced by serum levels of 4kD FITC dextran following gavage (Figure 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 (Figure 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 (Figure S3), acquired less luminally administered fluorescent Ova (Figure 2B and 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 (Figure 2A) and gavaged fluorescent Ova was not found leaking between SI epithelial cells or in the lamina propria of goblet cell deficient mice (Figure S2E). Isolation of the CD103+ 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 able to acquire gavaged Ova in a manner capable of inducing CD4+ T cell responses in *ex vivo* co-cultures with Ova specific T cells from OTII T cell receptor (TCR) transgenic mice when goblet cells and GAPs were absent (Figure 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 (Figure S4A) and no defect in induction of T cell proliferation when exogenous Ova was added to *ex vivo* co-cultures (Figure S4B). We attributed the decrease in LP-APC antigen acquisition to the loss of goblet cells and GAPs, as we saw very few intestinal enteroendocrine cells and few Paneth cells acquiring luminal Ova in wildtype mice (Figure 1A and B), and Paneth cells were still present at this time following deletion of Math1 (Figure 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 (Figure 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 (Figure 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 OTII Rag−/− T cells in the MLN in response to Ova in mice lacking goblet cells and GAPs when compared with their Cre-littermates (Figure S4D). We also observed that GAPs were decreased in the distal colon of mice lacking goblet cells (Figure 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 (Figure 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* (Figure 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 (mEGF) significantly impaired LP-APC capture of luminally administered fluorescent Ova (Figure 3A and 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 (Figure 3C). Moreover, mEGF significantly impaired antigen specific CD4+ T cell responses to oral Ova *in vivo* in the MLN (Figure 3D). Importantly, deletion of the EGFR in goblet cells using an inducible Math1 driven Cre recombinase, $EGFR^{f/f}Math1^{Cre*PR}$ mice, reversed the effects of mEGF on GAP inhibition, and T cell responses to luminal Ova in *ex vivo* cultures and *in vivo* (Figure 3A, C, and D), demonstrating that the defect in antigen capture could not be attributed to effects of EGF 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, ($mAChR4^{f/f}Math1^{Cre*PR}$ mice) did not affect goblet cell numbers (Figure 3E), but impaired GAP formation (Figure 3F). Unlike the deletion of goblet cells, we did not see an increase in leak when GAPs were inhibited (Figure 3G) and accordingly we did not see a reduction in the mucus barrier when GAPs were inhibited and goblet cells remained intact (Figure S5A and B). Inhibition of GAPs by deletion of the mAChR4 on goblet cells impaired luminal fluorescent Ova acquisition by LP-APCs (Figure 3H and I), and impaired antigen specific CD4+ T cell responses to gavaged Ova in the SI draining MLN (Figure 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 (Figure S6A), but were not inhibited by deletion of mAChR4 in goblet cells (Figure 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 (Figure 4A). This decrease largely affected the Helios- pTregs in the SI LP (Figure 4B and C). We observed little change in the Helios-pTreg population in the colon LP(Figure 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[39,40], almost all of these LP Helios- Tregs expressed the transcription factor RORγt (Figure 4B), which can be expressed by SI LP pTregs with specificity to dietary antigens[13]. Further we observed that the SI LP pTreg population was reduced with GAP inhibition by mEGF in a goblet cell intrinsic EGFR dependent manner (Figure 3A and 4D) and upon GAP inhibition by deletion of the mAChR4 in goblet cells (Figure 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 (Figure 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 LP-APC population as LP-APCs isolated from mice lacking goblet cells were impaired at inducing antigen specific pTregs in *ex-vivo* cultures (Figure 4G).

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 (Figure 5A). Accordingly, the absence of goblet cells and GAPs resulted in a decrease in the IRF4+ SI LP-APC population (Figure 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 (Figure 5C). CD103+ CD11b− and CD103+ CD11b+ SI LP-DCs can have aldehyde dehydrogenase activity [7,45], which facilitates the production of all-trans retinoic acid, a factor promoting the differentiation of and imprinting of pTregs with gut homing molecules [46–48]. We observed that in the absence of goblet cells, SI LP-DCs had reduced aldehyde dehydrogenase (ALDH) activity (Figure 5D) and an impaired ability to induce the expression of the gut homing molecules α4β7 and CCR9 on responding T cells in *in vitro* co-cultures (Figure 5 E and 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 (Figure 5G) and by GAP inhibition via the deletion of mAChR4 in goblet cells (Figure 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 [11]. 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+ MHCII+ CD11b+ F4/80+ LP-APCs (Figure 5I and 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 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, challenged with Ova in the footpad, and evaluated for footpad swelling 24 hours 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 (Figure 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 (Figure 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 EGF 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 (Figure 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 (Figure S7A). However we did not see an increase in monocytes in the lamina propria when GAPs were inhibited and goblet cells remained intact (Figure S7B) 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 (Figure 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 (Figure 6 A–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 [49–51]. 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 naive T cells into Foxp3 expressing pTregs [52,53], which is facilitated by a local environment containing all-trans retinoic acid (ATRA) and TGFβ [46,47,54,55]. Within the gut CD103+ DCs and MLN stromal cells expressing retinaldehyde dehydrogenase, the enzyme necessary to convert retinal to the biologically active ATRA, are sources of ATRA supporting pTreg induction and imprinting gut homing molecules on lymphocytes [46,56–60]. DC imprinting with retinaldehyde dehydrogenase activity is induced by luminal retinoids and by DC association with the intestinal epithelium [45,61,62]. 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 [63], and select members of the gut microbiota promote pTregs through bacterial products or metabolites [42,64–70]. 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 [71–73]. 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 [16,74], 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 [24], 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[15], CD4+ T cell responses to luminal antigens are not [4,15], suggesting that the defect in oral tolerance in CX_3CR1 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 [15,25]. 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 CX_3CR1+ LP-APCs[75] 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 CX_3CR1+ LP-APCs [26], or in the distal colon, the site where tolerance to luminal antigen is induced in the colon [14]. 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 Peyer's patches, however subsequent studies identified M cells overlying the non-follicle bearing villous epithelium[18]. Villous M cells are rare in the steady state but can be induced by systemic treatment with TNF superfamily member receptor activator of NF-κβ ligand (RANKL), whose expression is normally restricted to subepithelial stromal cells restricted to the Peyer's patches [76]. These villous M cells can be closely associated with mononuclear cells and have the capacity to transcytose bacteria to induce immune responses to luminal bacteria [18,77]. 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. Similarly, 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 [27], 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 villus 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 life span, 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 are absent [20,24]. Indeed, we observed that in the absence of goblet cells and GAPs luminal antigen capture by CD103+ CD11b+, CD103− CD11b+, and 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 [20]. These studies focused on the functional outcome of inducing antigen specific T cell responses to luminal antigen, which was largely limited to the CD103+ LP-DC population and was impaired in the absence of goblet cells[20]. However other APC populations could acquire luminal antigen and stimulate antigen specific T cell responses when GAPs were induced above baseline levels[20], 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 [78,79]. 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 [4] or may be due to passage of antigen from CD103− LP-APCs to CD103+ LP-DCs [26]. 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 [45], which promotes the induction of pTregs [4,46,47,63]. Further, the production of IL-10 by resident LP macrophages supports the expansion and maintenance of pre-existing LP pTregs specific for dietary antigens [11]. 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 [20]. Combined with observations that the goblet cell protein mucin 2 imprints DCs with ALDH activity [63], 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 [8,14]. 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 regressed 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[80], 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[21,78,81], and that GAPs promote the induction of antigen specific tolerance to commensal bacteria during a defined pre-weaning

interval [41], the observations presented here suggest that goblet cell and GAP dysfunction may contribute to the pathogenesis of intestinal inflammatory diseases resulting from the loss of tolerance to dietary and microbial antigens. 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 mAChR4$^{fl/fl}$ 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[82], CD11c^{YFP} transgenic mice[83], CX$_3$CR1^{GFP} mice[84], Math1$^{fl/fl}$ mice[33], FoxP3^{GFP} mice[85], 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-Cre-ERT2) mice[86] were a gift from Sylvie Robine (Institut Curie, Paris, France). Math1$^{fl/fl}$ mice were bred to vil-Cre-ER^{T2} mice to generate mice with inducible depletion of goblet cells following deletion of Math1 in villin expressing cells. Math1$^{fl/fl}$vil-Cre-ER^{T2} mice and the injection protocol to induce goblet cell deletion have been previously described[21]. EGFR$^{fl/fl}$ mice[87] were a gift from Dr. David Threadgill, University of North Carolina. mAChR4$^{fl/fl}$ mice [88] were a kind gift from Jurgen Wess (National Institute Health, Bethesda, MD). EGFR$^{fl/fl}$ mice and mAChR4$^{fl/fl}$ mice were bred to Math1^{Cre*PR} mice [35] 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.

Intravital two-photon (2P) 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 hour, mice were sacrificed, and tissues thoroughly washed with cold PBS before fixing in 10% formalin buffered solution. Tissues were embedded in optimal cutting temperature compound (Fisher Scientific, Pittsburgh, PA) and 6 μm sections prepared. For studies in Figure 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 20x/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 16G plastic cannula inserted 3cm 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 minutes 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[45]. 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 2mg 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 16g plastic cannula was inserted 3 cm transanal into the colon. In some experiments, anesthetized mice were intraluminally treated with 10μg murine EGF (Shenandoah Biotechnology, Warwick, PA) 20 minutes 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, 10μg 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.

Adoptive 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 2μM CFSE (Invitrogen, Carlsbad, CA) and 2×10^6 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 16G plastic cannula as above. $EGFR^{fl/fl}$ or $EGFR^{fl/fl}Math1^{Cre*PR}$ mice, were administered with 10μg of murine EGF 20 minutes prior to receiving 15 mg ovalbumin in saline orally. Two days later SI draining MLNs or distal colon draining caudal and iliac LNs were removed and single-cell suspensions were prepared and analyzed by flow cytometry for CD45.1, CD3, Vβ5, Vα2 and CSFE. To evaluate the effect of systemic antigen administration on transferred T cells, 24 hours 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^{+} Foxp3^{GFP} OTII T cell receptor transgenic mice were flow cytometrically sorted for GFP^{-}, Vβ5^{+}, Vα2^{+}, CD45.1^{+}, CD62^{hi} cells. 5×10^{5} cells were *i.v.* administered into recipient Math1$^{fl/fl}$vil-Cre-ER^{T2} or Math1$^{fl/fl}$ 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 Foxp3^{GFP+} cells among the transferred cells. To evaluate the *de novo* generation of pTregs *in vitro* naive Foxp3^{GFP-} CD45.1 OTII T cells were isolated as above and cultured with flow cytometrically sorted LP-APCs at a ratio of 10:1 with 40μg of exogenous Ova. Five days later cultures were harvested and evaluated for Foxp3^{GFP+} 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 [45].

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 2μg 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 6μm 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 20g/L in drinking water, or drinking water alone for two weeks, or alternatively were gavaged with 20mg Ova daily for two weeks concurrent with gavage of 10μg murine EGF or given 25 mg Ova via enema. Two weeks and four weeks following dietary Ova exposure mice were immunized subcutaneously with 100μg Ova in incomplete Freund's Adjuvant (Sigma Aldrich). Two weeks after the last immunization mice were challenged with 20μg Ova in the footpad and the change in footpad thickness evaluated using measurements taken with micrometer calipers before and 24 hours after challenge. Blood was collected 24 hours after footpad challenge and serum levels of IFNγ were measured using Mouse IFNγ 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 posttest with correction for multiple comparisons for studies involving 3 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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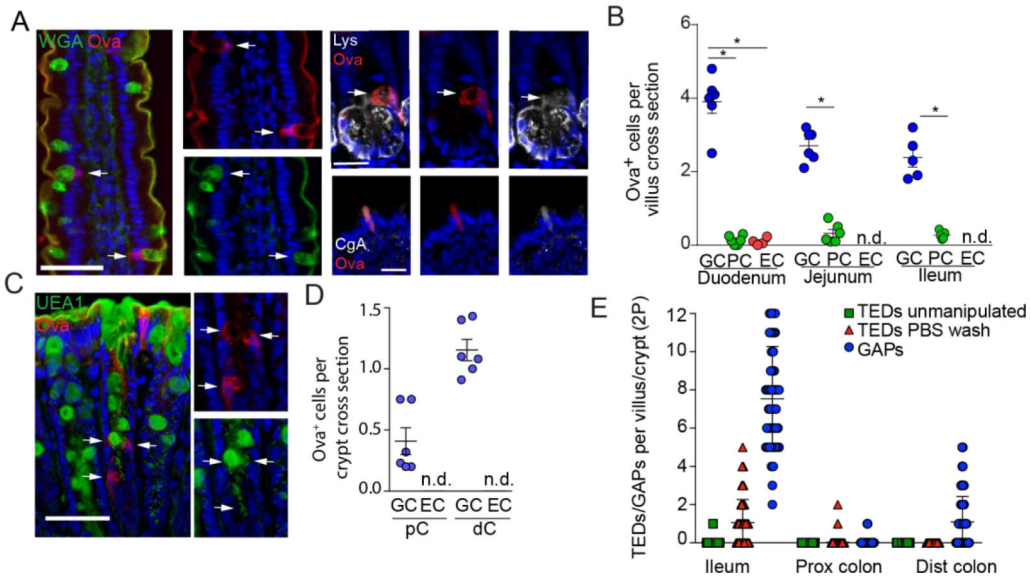

Figure 1: Goblet cell associated antigen passages (GAPs) are present at the sites of antigen acquisition where tolerance to luminal substances is induced in the steady state.

A) representative images and B) quantification of goblet cells (GC; wheat germ agglutinin (WGA)+ in SI), Paneth cells (PC; lysozyme (Lys) +), and enteroendocrine cells (EC; chromogranin A (CgA) +) taking up luminal fluorescent ovalbumin (Ova) in regions of the SI determined by immunofluorescent staining on fixed tissue sections. C) Representative images and D) quantification and of goblet cells (Ulex Europaeus Agglutinin I (UEAI) + in colon) and ECs taking up luminal fluorescent Ova in the proximal colon (pC) and distal colon (dC) as determined by immunofluorescent staining of fixed tissue sections. E) Quantification of TEDs and GAPs per SI villus or colon crypt obtained via *in vivo* two photon imaging. Scale bar = 50μm in large panels A and C and 20μm in small panels A, Each data point represents an individual mouse with 30 or more villi and 40 or more crypts evaluated per mouse in panels B and D. Each data point in panel E represents an individual crypt or villus. Data is presented as the mean +/− SEM. * = $P < 0.05$, ns = not significant, n.d. = not detected

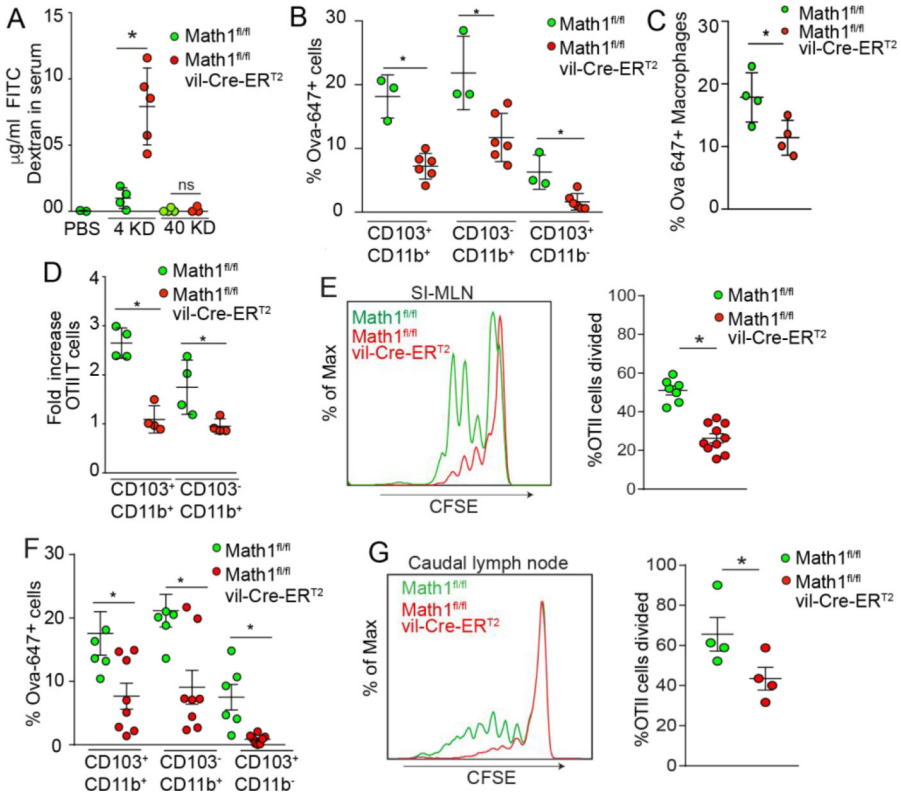

Figure 2: Goblet cells support antigen presenting cell acquisition of luminal antigen and CD4+ T cell responses to luminal antigen in the gut draining lymph nodes.

A) 4kD and 40kD FITC-dextran in serum after oral gavage in Math1$^{f/f}$vil-Cre-ER^{T2} mice and Math1$^{f/f}$ littermate controls. B and C) Luminal Ova acquisition by SI LP-APCs assessed by flow-cytometric analysis two hours post oral gavage. D) Antigen presentation capacity of SI LP-APCs isolated from mice Math1$^{f/f}$ and Math1$^{f/f}$vil-Cre-ER^{T2} mice given luminal Ova as assessed by expansion of Ova specific OTII T cells in *ex vivo* cultures. E) Histograms and quantification of *in vivo* proliferation of CFSE labeled OTII T cells in SI draining MLN of Math1$^{f/f}$ and Math1$^{f/f}$vil-Cre-ER^{T2} mice 2 days after oral Ova gavage. F) Antigen acquisition by distal colon LP-APCs assessed by flow cytometry 2 hours following intra-colonic administration of fluorescent Ova. G) Histograms and quantification of *in vivo* proliferation of CFSE labeled OTII T cells in distal colon draining caudal LN of Math1$^{f/f}$ and Math1$^{f/f}$vil-Cre-ER^{T2} mice 2 days after Ova enema. Data are representative of two or more replicates with ≥ 3 mice per group, each data point represents an individual mouse. Data is presented as mean +/− SEM, * = $P < 0.05$, ns = not significant.

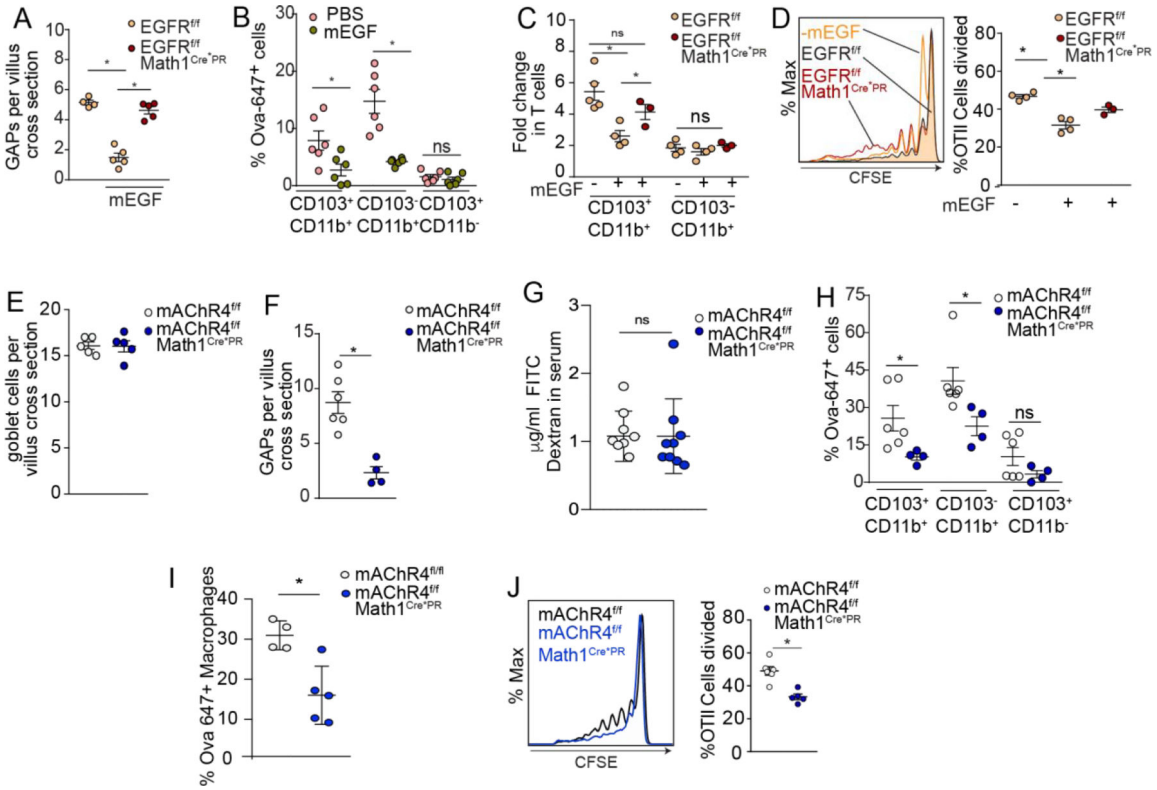

Figure 3: The GAP function of goblet cells supports the acquisition of, and CD4+ T cell responses to, luminal antigen.

A) GAPs per villus as assessed by immunofluorescent staining B) luminal fluorescent Ova capture by LP-APCs as assessed by flow cytometry C) ability of LP-APCs to stimulate Ova specific T cells *ex vivo* in response to luminal Ova and D) ability of Ova specific T cells to expand *in vivo* in response to luminal Ova in wildtype mice (panel B) and in mice lacking EGFR in goblet cells ($EGFR^{f/f}$ $Math1^{Cre*PR}$ mice) and littermate controls given EGF to inhibit GAPs. E) Goblet cells per villus as assessed by WGA staining, F) GAPs per villus as assessed by luminal fluorescent Ova uptake, G) FITC-dextran (4kD) in serum after oral gavage, H and I) luminal fluorescent Ova capture by LP-APCs as assessed by flow cytometry and J) ability of Ova specific T cells to expand *in vivo* in response to luminal Ova in mice lacking mAChR4 in goblet cells ($mAChR4^{f/f}$ $Math1^{Cre*PR}$ mice) and littermate controls. * = P< 0.05, ns = not significant, data presented as the mean +/− SEM. Each data point represents an individual mouse.

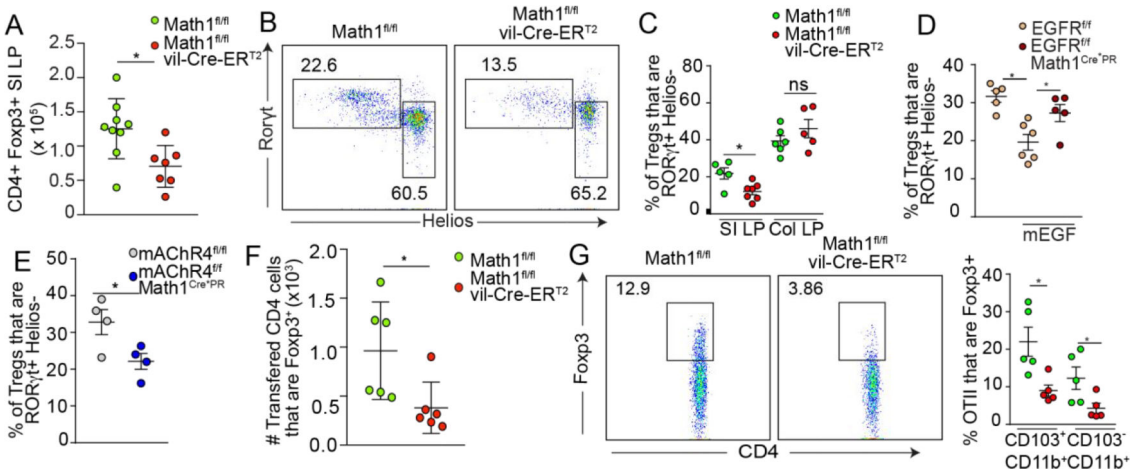

Figure 4: GAPs support the maintenance and induction of pTregs.

A) Absolute numbers of SI LP Tregs, and B) flow cytometry dot plots of Helios and RORγt expression by CD4+ Foxp3+ T cells in the SI LP, and C) quantification of RORγt+ Helios- pTregs populations in the SI and colon LP of goblet cell deficient mice (Math1$^{f/f}$ vil-Cre-ER^{T2} mice) and littermate controls. Quantification of SI LP RORγt+ Helios- pTreg populations in D) mice lacking EGFR in goblet cells (EGFR$^{f/f}$Math1^{Cre*PR} mice) and littermate controls treated with vehicle or mEGF, and in E) mice lacking mAChR4 in goblet cells (mAChR4$^{f/f}$ Math1^{Cre*PR} 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 is presented as the mean +/− SEM. Each data point represents an individual mouse.

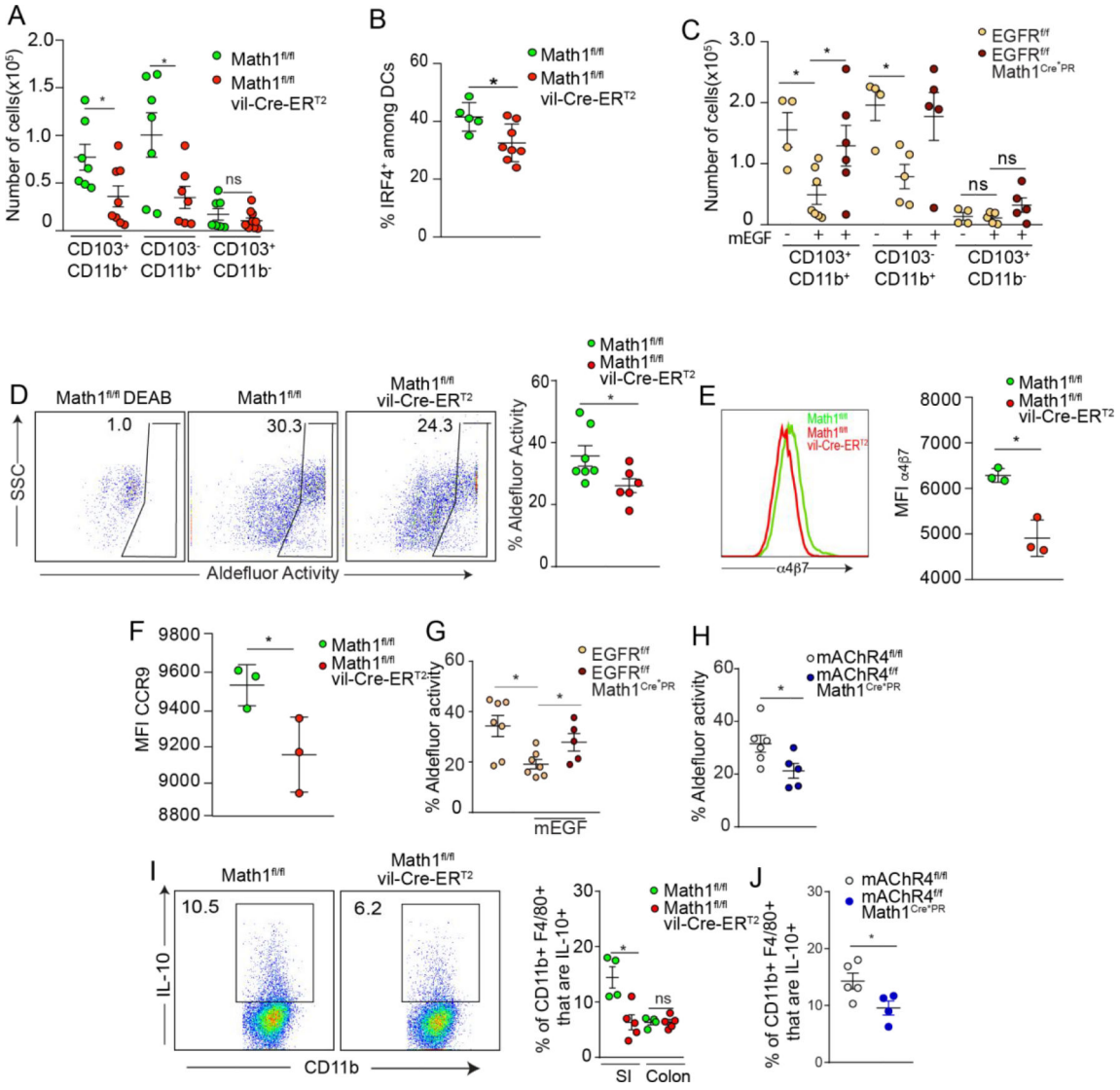

Figure 5: GAPs support the imprinting of LP-APCs.

Quantification of A) SI LP-APC subsets and B) IRF4+ SI LP-DCs in goblet cell deficient mice (Math1$^{f/f}$ vil-Cre-ER^{T2} mice) and littermate controls. C) Quantification of SI LP-APC subsets in mice lacking EGFR in goblet cells (EGFR$^{f/f}$Math1^{Cre*PR} mice) and littermate controls treated with mEGF. D) Flow cytometry dot plots and quantification of aldehyde dehydrogenase (ALDH) activity in LP CD11c+ MHCII+ SI APCs in goblet cell deficient mice (Math1$^{f/f}$ vil-Cre-ER^{T2} mice) and littermate controls. Expression of gut homing molecules E) α4β7 and F) CCR9 on OTII T cells following three days of *in vitro* culture with Ova and CD103+ DCs isolated from goblet cell deficient mice or littermate controls. Quantification of SI LP-APC with ALDH activity in G) mice lacking EGFR in goblet cells (EGFR$^{f/f}$Math1^{Cre*PR} mice) and littermate controls treated with mEGF and in SI LP-APCs from H) mice lacking mAChR4 in goblet cells (mAChR4$^{f/f}$ Math1^{Cre*PR} mice) and littermate controls. I) Flow cytometry plots of SI LP macrophages and quantification of IL-10 expression by LP CD45+ CD11c+ MHCII+ F480+ cells from mice lacking goblet cells and their littermate controls. J) Quantification of IL-10 expression by SI LP

macrophages from mice lacking mAChR4 in goblet cells and their littermate controls. * = $P < 0.05$, ns = not significant. Data is presented as the mean +/− SEM. Each data point represents and individual mouse, with the exception of E and F, where LP-APCs were pooled from three goblet cell deficient mice or three littermate controls.

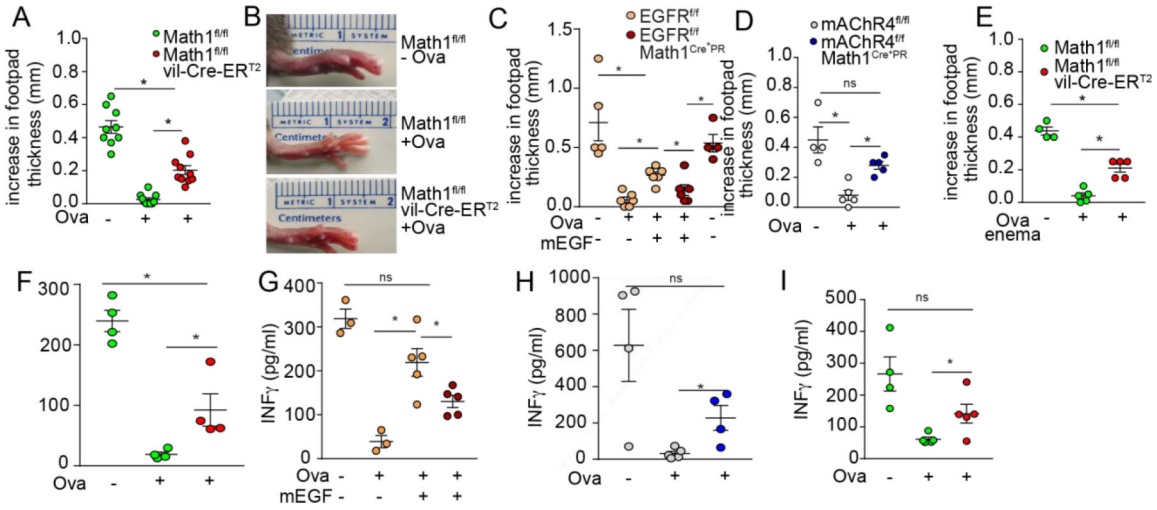

Figure 6: GAPs support tolerance to dietary antigen and tolerance to luminal antigens in the distal colon.

A) Quantification and B) images of footpad swelling following the induction of oral tolerance by dietary Ova, Ova immunization, and Ova footpad challenge in mice lacking goblet cells (Math1$^{f/f}$ vil-Cre-ER^{T2} mice) and their littermate controls. Quantification of footpad swelling following dietary Ova, Ova immunization, and Ova footpad challenge in C) mice lacking EGFR in goblet cells (EGFR$^{f/f}$Math1^{Cre*PR} mice) and their littermate controls treated with mEGF and in D) mice lacking mAChR4 in goblet cells (mAChR4$^{f/f}$ Math1^{Cre*PR} mice) and littermate controls. E) Quantification of footpad swelling following the induction of tolerance by Ova enema, Ova immunization, and Ova footpad challenge in mice lacking goblet cells (Math1$^{f/f}$ vil-Cre-ER^{T2} mice) and their littermate controls. F-I) Serum IFNγ in mice treated as in A-E 24 hours following footpad challenge. * = $P < 0.05$. Data is presented as the mean +/− SEM. Each data point represents an individual mouse.