The Role of the Calcium-Sensing Receptor in Gastrointestinal Inflammation

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Abstract

The gastrointestinal (GI) tract must balance the extraction of energy and metabolic end-products from ingested nutrition and resident gut microbes and the maintenance of a symbiotic relationship with this microbiota, with the ability to mount functional immune responses to pathogenic organisms to maintain GI health. The gut epithelium is equipped with bacteria-sensing mechanisms that discriminate between pathogenic and commensal microorganisms and regulate host responses between immunity and tolerance. The epithelium also expresses numerous nutrient-sensing receptors, but their importance in the preservation of the gut microbiota and immune homeostasis remains largely unexplored. Observations that a deficiency in the extracellular calcium-sensing receptor (CaSR) using intestinal epithelium-specific receptor knockout mice resulted in diminished intestinal barrier integrity, altered composition of the gut microbiota, modified expression of intestinal pattern recognition receptors, and a skewing of local and systemic innate responses from regulatory to stimulatory, may change the way that this receptor is considered as a potential immunotherapeutic target in gut homeostasis. These findings suggest that pharmacologic CaSR activators and CaSR-based nutrients such as calcium, polyamines, phenylalanine, tryptophan, and oligo-peptides might be useful in conditioning the gut microenvironment, and thus, in the prevention and treatment of disorders such as inflammatory bowel disease (IBD), infectious enterocolitis, and other inflammatory and secretory diarrheal diseases. Here, we review the emerging roles of the CaSR in intestinal homeostasis and its therapeutic potential for gut pathology.

Keywords: calcium-sensing receptor; diarrhea; intestinal inflammation; intestinal permeability; microbiota; polyamines

1. Introduction

A crucial function of the intestinal epithelial monolayer is to act as a physical and chemical barrier. At the same time, this tissue must also allow for the selective transport of solutes and water between compartments. Transepithelial transport in the gut occurs via two routes: “transcellular” or transport through the cell, crossing the apical and basolateral plasma membranes; and “paracellular” or transport in between cells [1]. Special adaptations of the mammalian intestinal epithelium allow it to accomplish two outwardly incompatible roles in regards to its role as this barrier: (i) maintaining homeostasis with the symbiotic bacteria of the resident gut microbiota without inducing pathogenic inflammation and (ii) mounting a controlled, but effective defense from gastrointestinal (GI) pathogens [2, 3]. The ability of the epithelial monolayer to act in this capacity is mediated by the formation of a nexus of tight junctions (TJs) that regulate mucosal barrier integrity and paracellular permeability, the production of a mucus layer covering the lumen of the intestine, and the recognition of pathogens and production of antimicrobial peptides for effective immunity [4]. In fact, structural changes and/or alterations of any of the aforementioned characteristics are strongly associated with the development of inflammatory bowel disease (IBD) or chronic inflammation of the gastrointestinal (GI) tract, including Crohn’s disease and ulcerative colitis [5].

When the ability of the host to limit the resident microflora’s egress from the intestinal lumen is compromised, some species can invade deeper tissues to cause disease. Moreover, shifts in the composition of the microbiota, whether generated by antibiotic treatment, invasive pathogens, or dietary changes, can disturb the balance of organisms in the resident gut flora, and alter the metabolic network of the collective symbionts to favor the outgrowth of potentially pathogenic constituents. Known as “dysbiosis,” these changes can perturb immune regulatory networks that normally dampen intestinal inflammation, and may contribute to immune-mediated disease directed against gene products of the microbiota [6]. Therefore, to understand GI mucosal barrier function and the diseases that result in or result from compromise of this barrier, it is essential to consider not only its structural components, but also the interactions of the bacteria and other luminal components with cells and receptors of the host. Experimental data have shown that any disturbance of this co-existence with intestinal symbionts can result in severe immunodeficiency and risk of a myriad of disease processes [3].

Estimated at approximately 10^{12} organisms, most of which are bacteria (although archaea and eukaryotes are also represented), and with the greatest density being in the distal ileum and colon [7], the resident microflora benefits the host by providing essential factors, increasing the host's ability to harvest nutrients from food, and by limiting available resources to potential pathogens that must outcompete well adapted and well established resident microbes for metabolic and physical niches [6]. In addition to short chain fatty acids (SCFAs) like acetate, butyrate, and propionate, which are the major end products of microbial fermentation of non-digestible carbohydrates that have received significant attention as pivotal candidates for microbiota-host signaling, gut bacteria also contribute an expansive array of metabolites including vitamins (e.g., vitamins B6 and B12, folic acid, etc.), polyamines, secondary bile acids, bioactive lipids, choline metabolites, indole derivatives, phenols, neurotransmitters and neurotransmitter precursors [8-10]. However, our appreciation for how this assortment of bacterial metabolites interacts with host cells and cellular receptors is currently at a very early stage, and further work will be needed to determine the effects of this crosstalk in different intestinal disease states [10].

2. The host-microbe relationship

The composition of the gut microbiota is characterized by pronounced variability in different individuals, with temporal and spatial variations in the microbial distribution from the esophagus to the rectum throughout an individual's life span. This variability arises primarily at the species and strain levels, whereas over 90% of the gut bacteria are members of just two phyla, the Bacteroidetes and Firmicutes; the relative proportions of which exhibit a continuum within the human population [11]. It is significant that host-microbe interactions within the colon differ in several aspects from those in the proximal gut. First, the colonic microbiota substantially exceeds that of the small intestine with a transition across the ileocecal valve of several log-fold difference in total bacterial numbers and in the degree of diversity [12]. Secondly, small intestinal mucus is an incomplete single-layered barrier, while direct contact by bacteria with the surface epithelium in the colon is restricted due to a characteristic mucus bilayer, comprising an inner firmly attached layer that is free of bacteria and a loose unsecured outer layer that is associated with intestinal symbionts [13]. There is also a functional barrier in the colon created by intestinal epithelial cells' (IECs) production of antimicrobial factors [2, 12, 14].

Further compartmentalization of the commensal microbiota is mediated by the immune system. Scant levels of translocation may occur from the proximal and distal gut, including transepithelial uptake by dendritic cells (DCs) and by microfold (M) cells overlying lymphoid follicles, but access to the systemic circulation is limited by the immune system with the mesenteric lymph nodes (MLNs) acting as sentinels and sites from which an acquired immune response may be controlled or intensified [12, 15, 16]. IECs along with innate and adaptive immune cells use pattern recognition receptors (PRRs) to sense or recognize conserved molecular patterns on potentially pathogenic microbes, which initiates activation of cytokine and chemokine signaling pathways, antimicrobial killing (e.g., reactive oxygen and nitrogen species, antimicrobial proteins, phagocytosis, and autophagy), and initiation of adaptive T- and B-lymphocyte responses [17] (**Fig. 1**). Host PRRs recognize different ligands and include the family of Toll-like receptors (TLRs), C-type lectins, nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs), and retinoic acid-inducible gene I-like receptors (RLRs) [17].

2.1 Intestinal permeability versus the intestinal barrier

The physical “intestinal barrier” is the term used by gastroenterologists, microbiologists, and immunologists referring to the four layers of the intestinal wall-the mucosa, the submucosa, the muscularis, and the serosa; the vascular endothelium, and the mucus layer. In addition to this physical barrier, chemical substances also participate in barrier function, and comprise digestive secretions, cytokines, chemokines, inflammatory mediators, and antimicrobial peptides. The intestinal microbiota is integrally involved in metabolic and immune processes, and thus, modulates the physical barrier [3]. “Intestinal permeability” is the term used preferentially by electrophysiologists studying epithelial permeability using Ussing chambers and intestinal tissue explants to test the ability of molecules to cross epithelia and enter the submucosae in a research setting [18, 19]. Thus, “intestinal permeability” can be understood as a measurable feature of the “intestinal barrier” [3]. The integrity of TJs and transepithelial permeability are regulated by commensal microbial signals, including TLR2-dependent redistribution of TJ proteins to apical cell-cell contacts [20]. Thus, the capacity of IECs to sense and recognize surrounding microbes plays a pivotal role in their ability to regulate barrier function [4].

Chemically-induced colitis likely represents the most highly utilized animal model to induce intestinal inflammation [21]. A prototypical example is colitis induced by dextran sodium sulfate (DSS), which is orally administered to experimental mice in their drinking water. This chemical damages the colonic epithelium, resulting in disruption of barrier integrity and subsequent luminal antigen/bacterial translocation into the underlying mucosal immune system [22, 23]. Mice given DSS develop increased intestinal permeability before the onset of colonic inflammation. To this end, changes in TJ assembly occur before the inflammatory stage of this model, such as loss of zona occludens-1 (ZO-1) expression and alterations in occludin expression in colonic epithelia [24]. The ensuing inflammation is acute in nature, primarily consisting of neutrophilic and lesser macrophagic infiltration with concurrent expression of associated cytokines. Despite the artificial nature for initiating gut inflammation, chemically-induced models of colitis emphasize the significance of epithelial barrier disruption, which is likely the initial event resulting in the development of colonic inflammation, characteristic of these models [23].

3. The numerous roles of the calcium-sensing receptor

The extracellular calcium-sensing receptor (CaSR) is extremely multifaceted due to its ability to participate in various different signaling pathways that are ligand and tissue specific, enabling this receptor to play a variety of critical roles in the physiology and pathophysiology of both calcium regulation and other cellular functions which appear unrelated to calcium homeostasis [25]. First identified in 1993 by Brown et al., the CaSR was the first heptahelical guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) that was identified as having an ion, Ca^{2+} , as its physiological ligand. The main function of the CaSR is the control of the concentration of extracellular free ionized Ca^{2+} (Ca^{2+}_o) by regulating parathyroid hormone (PTH) secretion from chief cells of the parathyroid glands [26, 27]. More recent evidence suggests that the CaSR located in intestinal epithelial cells may also regulate calcium absorption by modifying responsiveness to 1,25-dihydroxyvitamin D3 (the hormonally active form of Vitamin D) independently of PTH [28].

In addition to its expression within bones, kidney, and parathyroid gland, the CaSR can be found in the lung, breast, liver, placenta [29], throughout the vasculature, and in the gut. In these tissues, the CaSR acts as a chemosensor, merging signals from nutrient availability (ions, L-

amino acids, sugars), acidification, salinity, and the presence of ubiquitous polyamines (organic compounds that have two or more primary amino groups). Altered CaSR expression and/or activity is associated not only with disorders of the parathyroid glands, but also with other conditions like vascular calcification, osteoporosis, and neoplasia [29]. A better understanding of the myriad of stimuli for this receptor and of the resulting tissue-specific signaling responses is critical to appreciating the non-calcitropic roles of the CaSR in health and in disease [30].

The CaSR functions as a general sensor of the extracellular milieu due to allosteric modification of Ca^{2+} affinity and efficacy by polyamines, L-amino acids, and certain oligopeptides [31]. Accordingly, the CaSR must function in the chronic presence of agonists, unlike most other GPCRs. This is possible because the CaSR adopts unusual mechanisms regulating receptor expression, trafficking, and degradation [32]. For example, instead of inducing internalization and causing desensitization in other GPCRs, continuous elevation of the primary agonist or the addition of allosteric activators increases the number of plasma membrane-localized CaSRs and sensitizes receptor function [32]. Also, in contrast to many other GPCRs that exist in either an “on” or “off” conformation, the CaSR likely adopts multiple active conformations stabilized by different agonists to generate a set of distinct intracellular signals and biological effects [33]. Consequently, super-agonism (more than 100% efficacy) and biased-agonism (selective activation or inactivation of one function over others) may occur when a combination of different agonists are used to influence the receptor function. This is an important distinction because these unusual properties could enable evaluation of different agonist combinations to design novel therapies that produce maximal therapeutic and minimal unwanted outcomes [34].

3.1 Signaling pathways utilized by the CaSR

Upon agonist stimulation, the CaSR can couple to the $G_{q/11}$, $G_{i/o}$, and $G_{\alpha_{12/13}}$ families of G proteins, consequently activating a variety of enzymes and cell signaling pathways including phosphatidylinositol-specific phospholipase C (PI-PLC), and subsequently, PKC and intracellular Ca^{2+} mobilization; MAPKs (ERK1/2, p38 MAPK, JNK); phospholipase A2 (PLA2), leading to arachidonic acid production; phospholipase D (PLD); and phosphatidylinositol 3-kinase (PI3K), leading to activation of the Akt pathway [25]. Since $G_{\alpha_{12/13}}$ proteins have been implicated in many different processes, including cell migration [35], it has been postulated that

CaSR-dependent signaling in breast and prostate cancer cells could be involved the metastatic spread of tumors [29, 36]. In some situations, the CaSR couples to $G_{i/o}$, suppressing adenylyl cyclase resulting in reduced cAMP levels. CaSR activation of the ERK1/2 pathway can also transpire via epidermal growth factor receptor (EGFR) stimulation, and CaSR activation through G_q stimulates serum response element (SRE)-mediated transcription through a Rho-dependent pathway [37, 38]. It has also been shown that the CaSR can couple to the G_s family in human pituitary and breast cancer cells to activate cAMP production and induction of hormone secretion [25, 39, 40].

4. The CaSR in the GI tract

The CaSR is found in many other tissues not directly involved in Ca^{2+} homeostasis, including along the majority of the GI tract. Receptor transcripts and/or protein have been detected in the stomach, small intestine, the colon, and with the underlying Meissner and Auerbach neural plexuses involved in smooth muscle function and intestinal motility [41]. The enteric nervous system (ENS) is composed of two inter-connected neural plexi: the submucosal Meissner's plexus that mainly controls fluid secretion by the epithelium, and the myenteric Auerbach's plexus that primarily controls gut motility. Both plexi contribute to diarrhea formation when they are disturbed. In humans and rodents, at least 50% of the fluid secreted in cholera, rotavirus, and other forms of secretory diarrhea are caused by activation of the ENS [42-45]. This activated ENS-evoked secretion is also present in patients with irritable bowel syndrome (IBS) [46], IBD [47], and intestinal allergies.

The CaSR has been identified on both the apical and basolateral membranes of villus cells in the small intestine [48], and of surface and crypt epithelial cells in the colons of rodents and humans [41, 48, 49]. CaSR activators modulate intestinal motility (Cheng et al., unpublished observations), inhibit Cl^- secretion [49-52], and stimulate Na^+ absorption in both the small intestine and colon [53, 54]. These findings are in line with the primary functions of the colon, which are to absorb and secrete fluid, contributing to the homeostasis of salt and water balance. Fluid absorption and secretion in the intestine are highly regulated; in adult humans, approximately 9.5 L of fluid is secreted and then absorbed daily throughout the GI tract [55]. Thus, mechanisms that alter salt, and consequently fluid absorption, can rapidly alter fluid homeostasis into net fluid secretion. Diseases such as rotavirus infection in infants, IBD, and

neurogenic stimuli, as with IBS, all result in secretory diarrhea. With the exception of the mechanisms that cause cholera, we still know very little about epithelial and enteric nervous system determinants of fluid homeostasis in the colon [53]. A more thorough insight into the role of the CaSR in this intricate physiologic process will likely be pivotal to the comprehension of fluid homeostasis that significantly impacts regulated immunity and symbiosis with gut microbiota controlling unwanted gut proinflammation, which can result in intestinal pathology.

5. The CaSR in colorectal cancer

Activation of the CaSR by different ligands may stabilize the CaSR in unique activation states allowing preferential stimulation of different signaling pathways, referred to as “ligand-biased signaling” [56]. Modified CaSR signaling is associated with a number of pathophysiologic states; in neoplastic processes, CaSR expression either becomes reduced or even lost, or signaling pathways become activated that are different from those normally activated in their respective tissues [29]. The signaling may deviate from “normal” depending on the type of cancer, stage, grade, etc. Thus, there is need for a better understanding of how individual ligands affect CaSR-mediated signaling, and how this will result in the development of novel pharmaceutical therapies targeting the CaSR [29].

Recently, Rey and colleagues examined how variations in CaSR-mediated intracellular Ca^{2+} oscillation frequency and amplitude affect cellular physiology. In human colonic epithelial cells, the CaSR can induce two distinct oscillatory pathways: while CaSR-mediated high frequency (~ 3 to 4 min^{-1}) sinusoidal intracellular Ca^{2+} oscillations induce inhibition of proliferation, low frequency ($\sim 1.5 \text{ min}^{-1}$) transient intracellular Ca^{2+} oscillations do not [57]. Thus, the mechanisms that control intracellular Ca^{2+} oscillation frequency and amplitude are also critical for various CaSR-dependent biological responses [38].

Signaling pathways activated by GPCRs expressed in the GI tract play an important role in the regulation of multiple functions of the digestive system, including cellular differentiation, proliferation, inflammation, and the promotion of colorectal cancer (CRC) [58, 59]. In fact, the expression of the CaSR is greatly reduced or completely lost in CRC [60], indicating that signaling pathways activated by this receptor negatively control cellular proliferation and inhibit CRC development [59]. Recently, using a *Casr* intestinal-specific knock-out mouse [61], Rey et al. showed that genetic ablation of *Casr* leads to hyperproliferation of colonic epithelial cells,

enhanced β -catenin nuclear localization, and changes in crypt architecture. Mechanistic studies using NCMiCaSR, an immortalized cell line derived from normal human colon mucosal epithelium with functional APC and β -catenin proteins [62], demonstrated that CaSR stimulation promotes a decrease in the phosphorylation of β -catenin which corresponded with its redistribution to the plasma membrane and β -catenin-mediated transcriptional downregulation. These studies indicate that CaSR signaling negatively controls colonic epithelial cell proliferation *in vivo* through a mechanism that involves β -catenin phosphorylation [59].

6. The role of the CaSR in secretory diarrhea and colitis

To accomplish its functions, the normal colonic mucosa is organized as a sheet of epithelial cells with invaginations forming stereotypical crypts, which make up about 90% of the epithelial mass of the colon. Each crypt contains distinct functional compartments with stem cells at the base that are responsible for continuous renewal of aged epithelial cells, a proliferating transit amplifying zone extending to the mid crypt, and the most mature epithelial cells located at the crypt apex [63]. Under baseline conditions (in the absence of drugs, hormones, or other factors), the net fluid transport by colonic crypts is absorptive in nature. However, upon exposure to cell-permeable cyclic AMP (cAMP) analogs, forskolin (an herbal extract commonly used to raise levels of cyclic AMP in the study of cellular physiology isolated from *Coleus forskohlii*, a plant belonging to the mint family) or other agents that activate adenylate cyclase, or modifiers of cAMP metabolism such as phosphodiesterase (PDE) inhibitors, crypts change the direction of net fluid transport from absorption to secretion [41, 64]. The addition of cAMP-generating hormones/factors such as 5-hydroxytryptophan (5-HTP) or prostaglandin E₂ (PGE₂) to the blood/interstitial surface of the crypt epithelium also upregulates fluid secretion in the colon [65, 66]. When cyclic nucleotides (cAMP or cGMP) modulate these fluid transport processes, profound fluid and electrolyte losses from the colon with secretory diarrheas, such as cholera [66] result [41].

Using established CaSR agonists, Ca²⁺_o, gadolinium (Gd³⁺), and the antibiotic, neomycin, it has recently been found that both apical and basolateral CaSRs on crypt cells from proximal and distal colonic sections excised from rats and human biopsy specimens were functionally active [49]. In rat distal colonic epithelial cells, increasing Ca²⁺_o generated intracellular D-myo-inositol 1,4,5-trisphosphate (IP₃) and Ca²⁺, “typical” CaSR second messengers, and significantly

attenuated forskolin-stimulated net fluid secretion. These experiments demonstrated pathways modulating net intestinal fluid transport that could have important implications for the prevention and treatment of certain diarrheal diseases associated with elevated cAMP, such as cholera [49].

6.1 Polyamines and the CaSR

Polyamines (PAs) are small aliphatic polycations first described in 1678 by Antonie van Leeuwenhoek in seminal fluid, resulting in the naming of two of its members, spermine and spermidine. PAs are predominantly derived from the amino acids, ornithine and methionine, while arginine and lysine serve as alternative, secondary sources of these metabolites [67]. Polyamines are present in significant quantities in virtually all eukaryotic cells and extracellular fluids [68], can activate the CaSR [66], and are essential nutrients for postnatal growth and development of the small intestinal and colonic mucosae [69-71]. The polyamines, spermine and spermidine, and their precursor, putrescine, may play a pro-differentiation role in healthy colonocytes. The high normal proliferation rate of GI mucosal epithelial cells may explain their special demand for polyamines [50]. Although polyamines can be transported into cells and can influence many cellular processes [67], these polycationic bioamines are fully protonated in physiologic solutions so that they resemble the multivalent metal cations such as Ca^{2+} and Mg^{2+} . The potency of polyamines is directly proportional to charge (spermine > spermidine > putrescine), consistent with models of CaSR activation. Polyamines also function by potentiating the activation of the CaSR by Ca^{2+}_o [50, 66]. Hypothesizing that extracellular polyamines may influence colonocyte function by directly activating or modifying Ca^{2+}_o -mediated activation of the CaSR, it has been demonstrated that the interactions of polyamines and Ca^{2+}_o can modulate CaSR-mediated increases in intracellular Ca^{2+} and IP_3 in rat distal colonic epithelial cells. These data indicated that rat colonic epithelial cells are capable of sensing and responding to extracellularly added polyamines, and that this polyamine-sensing is brought about by modulation of Ca^{2+}_o activation of the colonic CaSR [50]. Additionally, the effect of spermine to modulate baseline and forskolin-stimulated net fluid transport in isolated rat colonic crypts was assessed. Polyamines enabled subthreshold Ca^{2+}_o to abolish forskolin-stimulated fluid secretion. An anti-secretory effect was also achieved by nanomolar concentrations of spermine at physiologic levels of Ca^{2+}_o , demonstrating the powerful effect of polyamines on colonic fluid transport. Thus, activation of either the luminal or basolateral CaSR by Ca^{2+}_o or a polyamine

such as spermine led to a reversal of forskolin-induced fluid secretion. These results support a role for the CaSR in mediating at least some of the effects of polyamines on colonic epithelial cells [50].

6.2 The CaSR and intestinal immunity

Recently, our laboratory also sought to clarify the role of the CaSR and the local and systemic consequences of its manipulation using intestinal epithelium-specific *Casr*^{-/-} mice [59]. Epithelial CaSR deficiency diminished intestinal barrier function, altered the composition of the microbiota, and skewed immunity towards a proinflammatory response [72]. Assessment of intestinal permeability was performed ex vivo using Ussing chambers; *Casr*^{-/-} mice exhibited reduced transepithelial resistance (TEER), and higher transepithelial conductance (G_T) and passive transport of FITC-conjugated dextran. Surprisingly, significant alterations in short-circuit current (I_{SC}) and ion transporter transcript measurements, indicative of defective transcellular transport, were not observed between the two groups, suggesting that the CaSR exclusively affects the paracellular transport pathway [72]. Consistent with a defective intestinal epithelial barrier, *Casr*^{-/-} mice also showed decreased colonic expression of TJ molecules, particularly occludin-2, a major component of TJs. *Casr*^{-/-} mice also demonstrated a significantly increased expression of myosin light-chain kinase-1, an enzyme that controls contractility of the perijunctional actomyosin rings and epithelial permeability [72]. Increased expression of the tight junction protein, *Cingulin*, was also seen in *Casr*^{-/-} mice compared to their wild-type counterparts [72]. Although the exact mechanism is unknown, *Cingulin* is associated with the expression of *Occludin*, *Claudin 2*, *Claudin 7*, and epithelial specific transcription factors, *Gata4*, *Gata6*, and *Hif-1 α* [73, 74], a feature also observed in *Casr*^{-/-} mice [72].

To evaluate if the breakdown of intestinal epithelial integrity in *Casr*^{-/-} mice altered the distribution of microbiota, a combination of real-time PCR and Illumina Miseq was utilized to analyze the microbiota of steady-state *Casr*^{-/-} and wild-type mice. No significant differences were seen between the overall richness and diversity of the two gut microbial communities. However, deeper analyses indicated significant changes in bacterial composition. At the phylum level, an outgrowth in the minor group *Deferribacteraceae* was noted, a group which was previously found to correlate with inflammatory responses in the colons of *Citrobacter rodentium*-infected mice [75] representing a model of bacterial-induced colitis. At the same time,

beneficial lactobacilli were decreased in *Casr*^{-/-} mice [72]. Furthermore, the relative abundance and distribution of the Gram-positive organism, *Clostridium coccoides*, was significantly altered in *Casr*^{-/-} mice, with depletion noted in the lumen and enrichment in the subepithelial layer. Consistent with enhanced bacterial translocation and dissemination in host tissues, *Casr*^{-/-} mice had significantly decreased epithelial expression of *Reg3β* and *Reg3γ* [72] encoding secreted C-type lectins that bind and protect against translocation and dissemination of Gram-negative [76] and Gram-positive bacteria [77, 78], respectively.

Speculating that dysbiosis may lead to pathogenic inflammatory immune responses locally, gene array analyses of the distal colon of wild-type and *Casr*^{-/-} mice revealed a marked increase in the expression of a range of PRRs and cytokine-encoding genes in the colons of *Casr*^{-/-} mice. Increased IL-1R expression was seen in colonic DCs in *Casr*^{-/-} mice, as well as in colonic CD4⁺ and CD8⁺ T lymphocytes. As further evidence of chronic intestinal inflammation and potential immunologic exhaustion in *Casr*^{-/-} mice, programmed cell death protein 1 (PD-1) expression was also significantly enhanced in colonic CD4⁺ and CD8⁺ T cells [72]. Moreover, *Casr*^{-/-} mice demonstrated more severe colitis with delayed recovery compared to their littermate counterparts, as assessed by weight changes and quality of the stools. Consistently, wild-type mice were able to recover from colitis, as demonstrated by the gross morphology and histopathology of the colons at day 13 post-DSS treatment. In contrast, *Casr*^{-/-} mice still displayed severe colonic inflammation and mucosal damage, indicating that the intestinal CaSR likely plays a key role in the regulation of induced inflammation, and may represent a potential therapeutic target for autoinflammatory disorders, including IBD [72].

B cells comprise nearly half of the lymphocyte population in the gut [79], where they perform diverse roles, including functioning as phagocytic and antigen-presenting cells, as well as differentiating into conventional antibody-secreting plasma cells. IECs express the polymorphic IgA receptor for the purpose of translocation of IgA into the gut lumen, which helps in the maintenance of different microbial constituents. Any perturbation in the relationship of IECs and B cells alters this homeostatic balance and often results in GI inflammation (**Fig. 1**). Mice deficient in PD-1 produce non-specific immunoglobulins [80], which result in altered microbiota and a phenotypic change in epithelial cell function [81]. These epithelial cells reduce their expression of GATA4, a signature transcription factor for their function in the gut [82]. Our analyses revealed a decrease in the expression of GATA4, an epithelial cell-specific

transcription factor along with a decrease in certain genes known to be dependent upon GATA4 [83, 84], *Alpi* and *Fabp2* [72]. Intestinal alkaline phosphatase (ALPI) detoxifies lipopolysaccharide (LPS) [85], whereas fatty acid binding protein-2 (FABP2, also known as intestinal-type fatty acid-binding protein (I-FABP) [84] is essential for the absorption of fatty acids from the gut lumen. Epithelial cells utilize these fatty acids as a primary energy source; these acids are also required for the expansion of various immunoregulatory cells, including regulatory T cells (Tregs). Thus, reduced GATA4 levels and its function in intestinal *Casr*^{-/-} mice may contribute to the overall inflammatory condition.

Colonic tissue isolated from *Casr*^{-/-} mice also showed a robust elevation in NF-κB-dependent genes, suggesting a potential role for CaSR-signaling in controlling the expression of these genes. cAMP-dependent signaling activates a group of strong transcriptional co-activating proteins, CBP/p300, to enhance the nuclear retention of c-Rel (a member of the NF-κB family of transcription factors). We hypothesize the CaSR-signaling leads to a decrease in CBP/p300 transactivators by reducing cAMP levels within the IECs. Additionally, increased cAMP levels in the *Casr*^{-/-} mice, led to the phosphorylation of Claudins 3 and 5 [86, 87] in a PKA-dependent fashion to disrupt the TJ, which we have observed in the absence of the CaSR (Sahay et al., unpublished observations).

B cells accumulate at the site of an epithelial breach to compensate for compromised barrier function. These B cells often produce higher levels of IgA and sometimes, IgG. A similar pattern was observed in the absence of the CaSR, where enhanced B cell numbers were found to accumulate in the colon with higher levels of IgA expression. Moreover, with increased systemic inflammation in CaSR-deficient mice, B cells in the periphery expressed CD93, a marker that is found on immature B cell precursors in the bone marrow, with much lower expression found in splenic B cells as they mature. It is re-induced during plasma cell differentiation and plays an important role in maintaining plasma cells in the bone marrow [88, 89], indicating the impact of the CaSR on these cells that maintain antibody secretion and potentially regulate the levels of plasma cells, not only in bone marrow niches, but also in the gut (**Fig. 2**). Thus, these data highlight the immunophysiologic role of the CaSR in sensing signals related to induced proinflammation, and how this functionally governs local and peripheral homeostasis that potentially keep the composition of gut commensals in check. Nonetheless, further studies are warranted to expand upon the influential role of the CaSR in controlling the composition of

healthy gut microbiota and their induced metabolites, all of which regulate immunity during steady state and inflammatory conditions.

7. Summary and future perspectives

Acute infectious diarrhea remains the number one cause of deaths in children around the world, even before the recent Ebola virus outbreak. It is estimated that 1.3 million children die each year, not due to infection per se, but as a result of the associated diarrhea and dehydration [90-92]. Although it is valuable for correcting dehydration, oral rehydration solution does not “halt” intestinal fluid loss and thus, does not reduce the severity or duration of diarrhea. It also does not treat the inflammatory component of diarrhea, and its use in treating diarrhea has dropped significantly by medical practitioners [93]. Diarrhea can be pathologically grouped into two types: secretory (as seen in cholera, travelers’ diarrhea, and rotavirus) and inflammatory (as observed in enterocolitis caused by *Salmonella* and *Shigella*, as well as IBD). The CaSR is expressed along the majority of the GI tract and also on inflammatory cells, as well as other cells that regulate inflammatory diarrhea [94, 95], suggesting a potential protective role in this setting.

Since emerging evidence has shown the importance of intestinal barrier function in maintaining gut immune homeostasis, increased efforts and investment in developing strategies by which to manipulate epithelial innate immunity and permeability have been made in order to achieve therapeutic effects in human disease. Presently, a number of approaches have been made and/or proposed to improve intestinal barrier function using several different strategies [23]. Such drug development targeting the CaSR could enhance epithelial innate immunity, gut homeostasis, decrease the permeability of the epithelial barrier, and/or decrease the likelihood of gut dysbiosis that can lead to or exacerbate IBD, infectious colitis, and secretory diarrhea. In particular, it will be critical to explore the role of innate and adaptive immune cells and the effects of the CaSR on their ability to protect the gut.

Figure Legends

Fig.1 Multiple effects of intestinal Calcium sensor receptor (*Casr*) deficiency. (Left panel) Expression of *Casr* on gut epithelial cells (EC) maintains barrier function and corresponding cellular functions. (Right panel) Mice lacking *Casr* on intestinal epithelial cells exhibit local, as well as systemic inflammation. Several factors contribute to this inflammation: (i) dysbiotic microbiota; (ii) breach of the intestinal barrier; (iii) translocation of microbes and their products into the lamina propria and systemic circulation; (iii) accumulation of inflammatory innate cells [e.g., dendritic cells (DCs)]; (iv) exhausted T cells; and (v) an increase in CD138⁺ B cells (plasma cells) and CD93⁺ B cells (immature B cells).

Fig. 2 Alterations in the B cell compartment of intestinal-specific *Casr*^{-/-} mice. Colonic cells were isolated from CaSR sufficient and *Casr*^{-/-} mice and the cells stained with fluorochrome-labeled antibodies against B220, IgA, CD138, CD45, CD21, CD23, MHC II and CD93. The stained cells were subjected to flow cytometric analyses. (A) Plasma cells; (B) Immature B cells. Data represent observations from two independent experiments and are shown as mean +/- S.E.M. (n = 10 mice/group). **P* < 0.05 compared to wild-type mice (CaSR sufficient).

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Figure 1

Fig.1 Multiple effects of intestinal Calcium sensor receptor (*Casr*) deficiency. (Left panel) Expression of *Casr* on gut epithelial cells (EC) maintains barrier function and corresponding cellular functions. (Right panel) Mice lacking *Casr* on intestinal epithelial cells exhibit local, as well as systemic inflammation. Several factors contribute to this inflammation: (i) dysbiotic microbiota; (ii) breach of the intestinal barrier; (iii) translocation of microbes and their products into the lamina propria and systemic circulation; (iv) accumulation of inflammatory innate cells [e.g., dendritic cells (DCs)]; (v) exhausted T cells; and (vi) an increase in CD138⁺ B cells (plasma cells) and CD93⁺ B cells (immature B cells).

Figure 2

Fig. 2 Alterations in the B cell compartment of intestinal-specific *Casr*^{-/-} mice. Colonic cells were isolated from CaSR sufficient and *Casr*^{-/-} mice and the cells stained with fluorochrome-labeled antibodies against B220, IgA, CD138, CD45, CD21, CD23, MHC II and CD93. The stained cells were subjected to flow cytometric analyses. (A) Plasma cells; (B) Immature B cells. Data represent observations from two independent experiments and are shown as mean +/- S.E.M. (n = 10 mice/group). **P* < 0.05 compared to wild-type mice (CaSR sufficient).