Epithelial CaSR deficiency alters intestinal integrity and promotes proinflammatory immune responses

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Abstract

The intestinal epithelium is equipped with sensing receptor mechanisms that interact with luminal microorganisms and nutrients to regulate barrier function and gut immune responses, thereby maintaining intestinal homeostasis. Herein, we clarify the role of the extracellular calcium-sensing receptor (CaSR) using intestinal epithelium-specific Casr−/− mice. Epithelial CaSR deficiency diminished intestinal barrier function, altered microbiota composition, and skewed immune responses towards proinflammatory. Consequently, Casr−/− mice were significantly more prone to chemically induced intestinal inflammation resulting in colitis. Accordingly, CaSR represents a potential therapeutic target for autoinflammatory disorders, including inflammatory bowel diseases.

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1. Introduction

The single layer of epithelium composing the intestinal mucosa acts as a barrier to impede the passage of toxins, pathogens, and foreign antigens, while selectively allowing the transport of electrolytes, essential nutrients, and water from the intestinal lumen into peripheral circulation [1]. Molecules pass through the intestinal epithelial monolayer by two routes: transcellular, through the apical and basolateral membranes of a cell; and paracellular, via the intercellular space between adjacent cells [2]. It is the function of apical junctional complexes to seal off the paracellular pathway of transport. The tight junction (TJ) and the adherens junction (AJ) comprise the apical junctional complex. In addition, desmosomes, located basolaterally beneath the AJ, strengthen the bond between adjacent epithelial cells by facilitating cellular proximity and TJ assembly [3]. These epithelial barriers are not absolutely impermeable, and intestinal epithelia have TJs with lower resistance than do other types of epithelia, such as that of the gallbladder, which compartmentalize irritating bile acids from the rest of the abdominal cavity [3]. Unfortunately, these low resistance TJs are prone to leakage, and emerging data suggest that the pathogenesis of inflammatory bowel disease (IBD) is related to three fundamental and self-perpetuating circumstances: compromised intestinal barrier function; exposure of intestinal luminal contents to leukocytes in the lamina propria; and uncontrolled inflammatory immune responses [1].

Importantly, it has recently been shown that the gut epithelium serves as a “communicator” between the luminal flora and the subepithelial immune system comprised of innate and adaptive immune components, including dendritic cells (DCs) and lymphocytes [4]. The epithelium expresses an array of pattern recognition receptors (PRRs), and disruption of these epithelium-derived bacteria-sensing/modulating mechanisms can result in uncontrolled
immune responses [5–9]. Resident gut commensal bacteria can also shape local mucosal and systemic immunity by providing critical signals that maintain gut homeostasis [10]. Previous studies have demonstrated that nutrients can modulate the composition of the gut microbiota, epithelial cell function, and host immunity [11–19]. For nutrient-dependent signaling, the gut epithelium is equipped with various nutrient-sensing mechanisms; however, their importance in gut bacteria-sensing, epithelial cell function, and immune homeostasis remains largely unknown.

One such nutrient-sensing receptor is the extracellular calcium-sensing receptor (CaSR) [20]. CaSR is a G protein-coupled nutrient-sensing receptor that is widely expressed in a range of tissues and species [21,22] to regulate calcium homeostasis [20] and osmotic balance [22–24]. Epithelial cells along the entire gastrointestinal (GI) tract express the CaSR [25–30]. Although previous investigations have studied the role of GI CaSR in fluid transport, intestinal balance [22–24], we focused here on the local and systemic consequences of alterations in CaSR signaling.

2. Materials and methods

2.1. Mice

C57BL/6 mice lacking Casr expression in intestinal epithelial cells (Casr−/−) and their Casr+/+ littermates were bred and maintained in-house at the University of Florida Communicore Animal Facility. Casr−/− mice were generated as previously described [31]. Briefly, Casr+/+ mice were bred with transgenic mice expressing Cre Recombinase under the control of the villin 1 promoter and genotyped prior to all experiments after a minimum of two generations. Mice were used at 5–10 weeks of age in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida.

2.2. Ex vivo transepithelial electrical resistance (TEER), short-circuit current (Isc), transepithelial conductance (Gt), and permeability measurements of intestinal tissues

Differences in electrogogenic ion transport in the colons of Casr+/+ and Casr−/− mice were quantified by measuring the short circuit current responses of freshly isolated colonic tissues mounted in modified Ussing chambers (Physiologic Instruments, San Diego, CA), as previously described [32,33]. Intestinal permeability was assessed using 4 kDa fluorescein isothiocyanate dextran, as described previously [34].

2.3. Lamina propria leukocyte (LPL) preparation

Colonic lamina propria (LP) cells were isolated as previously described [35], with minor modifications. Digestion buffer consisted of DMEM (GIBCO®, Life Technologies) containing 0.25 mg/mL collagenase type VII (Sigma–Aldrich), 0.125 U/mL Liberase TM Research Grade (Roche Applied Science, Indianapolis, IN), 10 mM HEPES, 0.1 M CaCl2 (Sigma–Aldrich), and 5% FBS (3 x 10 min digestions). Cells obtained from the digestions were combined and stained for flow cytometry-based analyses.

2.4. Flow cytometry and antibodies

Single cell suspensions obtained from processed spleens and MLNs, and LP lymphocytes were stained with LIVE/DEAD Aqua Dead Cell Stain Kit® (Molecular Probes®, Life Technologies). Mouse Fc Blocking Reagent (Miltenyi Biotec, Auburn, CA) was used prior to staining with combinations of the following antibodies or their corresponding isotype controls from eBioscience (San Diego, CA), Biologend (San Diego, CA), BD Pharmingen, or R&D Systems (Minneapolis, MN): CD45 (30-F11)-eFluor650NC, CD11c-(N418)-BV605, CD11b-(M1/70)-PE-Cy7, CD11b-(M1/70)-APC-Cy7, F4/80-(BM8)-PB, GR1-(RB6-8C5)-APC-Cy7, 1-A-I-E MHCII-(2G9)-FITC, 1-A-I-E MHCII-(2G9)-PE, CD3-(145-2C11)-APC-Cy7, CD4-(RM4-5)-BV605, CD8-(53-607)-PE-Cy7, PD-1-(29F.1A12)-BV421, Pro-IL-11-(JNTEN3)-APC, TNFα-(MP6-XT22)-PerCP-eFluor710, IL-6-(MP5-20F3)-FITC, IFNγ-(XMG1.2)-PerCP-Cy5.5, IL-17A-(TC11.18H10.1)-PE, FoxP3-(FJK-16A)-PE, IL-1R-(JAMA-147)-PE. Prior to intracellular staining, cells were fixed and permeabilized with BD Cytofix/ CytoPerm™ (BD Biosciences). A BD LSRFortessa™ (BD Biosciences) cell analyzer was used to acquire stained, fixed cells. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

2.5. Real-time PCR

For global gene expression changes, RNA was isolated from the tissues specified with Aurum™ Total RNA Kit (Bio-Rad). iScript™ Select cDNA Synthesis Kit (Bio-Rad) was used for reverse transcription and the obtained cDNA used for quantitative PCR by SYBR® Green Dye gene expression assay on a Bio-Rad CFX96™ Real time system; n = 8/group. The reaction was carried out in 10 μL final volume with an initial denaturation at 95 °C, followed by temperature cycling of 95 °C 30 s, 60 °C 30 s and 72 °C 30 s; 40 cycles were totally performed. For microbiota composition, ZR Fecal DNA Mini-Prep™ Kit (Zymo Research, Irvine, CA) was used to extract total fecal DNA per the manufacturer’s instructions. Real-time PCR analysis was performed on 2 ng of total DNA template (SsoAdvanced™ SYBR® Green Supermix, Bio-Rad) to target variable regions of bacteria group-specific 16S rRNA sequences [36]; n = 8/group. Groups were normalized to the housekeeper Eubacteria group to determine the relative abundance. Primers used can be found in Table S1.

2.6. 16S ribosomal DNA sequencing

Fecal DNA was amplified by illumina Miseq compatible primers, targeting the 16S rDNA V4-V5 region for microbiome analyses. Amplicons were purified by QIAquick Gel extraction kit (Qiagen, Madison, WI) and quantified with Qubit® 2.0 Fluorometer (Invitrogen, Grand Island, NY) and Kapa SYBR fast qPCR kit (Kapa Biosystems, Inc., Woburn, MA). Equal amounts of amplicons were pooled with 10% of Phix control. Miseq v2 reagent kit (illumina, Inc., San Diego, CA) was used to run the pooled samples on the Illumina Miseq. Data were analyzed as previously described [37].

2.7. Dextran sulfate sodium (DSS)-induced colitis

Casr+/+ and Casr−/− mice received 3% DSS in the drinking water for 7 days to induce colitis. Disease progression, including weight loss and diarrhea, was monitored throughout the study. Body condition scoring (BCS) of the mice, as determined by a veterinarian, was used as criteria for early termination of the experiment. Despite severe colitis, none of the mice reached a BCS requiring euthanasia. Mice were sacrificed at day 13 and the colons isolated for analyses. Tissues were fixed, sectioned, and stained with hematoxylin and eosin (H&E) by Histology Tech Services (Gainesville,
3. Results

3.1. Intestinal CaSR contributes to intestinal barrier function integrity

The evaluation of intestinal permeability ex vivo using Ussing chambers revealed that Casr−/− mice showed reduced transepithelial resistance (TEER), and higher transepithelial conductance (G)


denoted a significant increase in the expression of a range of PRRs and cytokine-encoding genes in the colons of Casr−/− mice (Fig. 3). To distinguish between proinflammatory responses in the intestinal epithelium due to attenuated CaSR signaling versus responses induced in resident immune cells, we examined colonic leukocytes by flow cytometry. Data show that colonic CD11b+ DCs upregulated their costimulatory molecules and its receptor in Casr−/− mice (Fig. 4A andB). As further evidence of chronic intestinal inflammation in Casr−/− mice, higher IL1R and programmed cell death (PD)1 were significantly expressed on CD4+ and CD8+ T cells (Fig. 5A andB).

We then investigated differences in systemic immune responses in these mice. We found a selective expansion of CD11b+ DCs in the mesenteric lymph nodes (MLNs) and spleens of Casr−/− mice (Fig. 5A and B). Moreover, higher numbers of Gr−1+CD11b+ neutrophils were noted in the spleens of Casr−/− mice (Fig. S1C). Similar to the colon, splenic CD11b+ DCs were populations can influence the other. To assess if the breakdown of intestinal epithelial integrity in Casr−/− mice alters the distribution of microbiota between either side of the epithelial barrier, we used a combination of real-time PCR and Illumina Miseq to analyze the microbiota of steady-state Casr−/− and wild-type mice. No significant differences were observed between the overall richness and diversity of the two gut microbial communities (Fig. 2A andB). However, deeper analyses indicated significant changes in composition. For instance, at the phylum level, we noted an outgrowth in the minor group Deferribacteraceae (Fig. 2C), which was previously found to correlate with inflammatory responses in the colons of Citrobacter rodentium-infected mice [38], a model of bacterial colitis. Concurrently, beneficial lactobacilli were decreased in Casr−/− mice (Fig. 2D). Moreover, the relative abundance and distribution of the Gram-positive Clostridium cocoides was significantly altered in Casr−/− mice, with depletion noted in the lumen and enrichment in the subepithelium (Fig. 2E).

Dysbiosis of the intestinal microbiota may lead to pathogenic inflammatory immune responses locally and systemically. Indeed, gene array analyses of the distal colon of wild-type and Casr−/− mice demonstrated a marked increase in the expression of a range of PRRs and cytokine-encoding genes in the colons of Casr−/− mice (Fig. 3).

3.2. Defect in epithelial CaSR signaling leads to gut microbe imbalance

A mutual interaction exists between the gut microbiota and the epithelial cells comprising the intestinal barrier, and both flora. Sections were analyzed/scored blindly by a boarded veterinary pathologist (JLO). Colitis was graded based on 7 parameters (0–17). Namely, degree of inflammation in the LP (0–3), goblet cell loss (0–2), abnormal crypts (0–3), crypt abscess (0–1), mucosal erosion and ulceration (0–1), submucosal spread to transmural involvement (0–3), and neutrophil numbers at 40× magnification (0–4). Stool consistency was scored as follows: 0 = normal, 1 = pasty, 2 = watery. 3 = watery with perianal staining.

2.8. Statistical analyses

Unless stated otherwise, representative data indicate mean ± S.E.M. Significance was determined by two-tailed unpaired t tests for two group comparisons (GraphPad Prism 5 for Mac OS X, La Jolla, CA).
Fig. 3. (1.5 columns) Increased inflammation in the colons of Casr−/− mice. Gene expression profile of the distal colons of Casr−/− and Casr+/+ littermate controls; n = 10 control mice and n = 20 Casr−/− mice. Data are shown as mean +/− S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 compared with WT. Heat maps were developed from the mean fold change in expression (Casr−/−/WT) calculated.

<table>
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<th>Phylum</th>
<th>p-value</th>
<th>Mean Abundance (%)</th>
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Fig. 2. (2 columns) Gut microbiota composition of Casr−/− mice. Microbiota composition alterations due to CaSR deficiency in healthy mice; n = 10 control mice and n = 20 Casr−/− mice. (A) Microbial evenness, diversity, and species richness in mice tested. Left: The species evenness index was calculated using the formula J = H/Hmax, where H is the Shannon diversity index and Hmax is the maximal value of H. Middle: The Shannon diversity index was used to estimate microbial diversity for each group. Right: The Chao richness index was used as a measure of species richness. (B) Unweighted UniFrac analyses were used to calculate distances between samples obtained from the two groups and three-dimensional scatterplots were generated by using principal coordinate analysis (PCoA). (C) Bacteria genera most enriched or depleted in Casr−/− mice, as measured by linear discriminant analysis (LDA). (D and E) Changes in abundance of specific bacteria and potential dissemination were measured by Real-Time PCR. Data are shown as mean +/− S.E.M. *P < 0.05, **P < 0.01 compared with control mice.

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significantly activated, as evident by proIL-1β production (Fig. S2A and B). In addition, IL-10+ was significantly reduced in splenic DCs of Casr−/− mice (Fig. S2B). DCs isolated from the MLNs of Casr−/− mice yielded similar trends, but no statistically significant differences (Fig. S3). Also, as in the colon, CaSR deficiency resulted in IL1R and PD1 upregulation in splenic and mesenteric CD4+ T cells (Fig. S4).

Intestinal IL1R signaling in CD4+ T cells has previously been found to promote Th17 responses [42]. We found that IL-17A+ as well as IFNγ+ CD4+ T cells were increased in the MLNs of Casr−/− mice (Fig. S5). Notably, despite the chronic intestinal inflammation observed in intestinal epithelium–specific Casr−/− mice, the frequency of FoxP3+ regulatory T cells (Tregs) was not decreased in these mice (Fig. S6); in fact, the number of Tregs was significantly increased in the MLNs of Casr−/− mice (Fig. S6B), perhaps as a compensatory mechanism to counteract the preexisting inflammation.

3.4. Casr−/− mice are more susceptible to DSS-induced colitis

Considering the notion that intestinal barrier dysfunction and imbalanced microbiota may contribute to enhanced susceptibility of Casr−/− mice to autoinflammatory diseases (e.g., IBD), the DSS acute induced colitis model was employed [43]. DSS promotes mucosal destruction independent of the intestinal microbiota, but the intestinal microbiota is thought to modify responsiveness and susceptibility to this chemical [44,45]. Casr−/− mice demonstrated more severe colitis with delayed recovery compared to their littermate counterparts, as demonstrated by weight changes and stool consistency at the endpoint of the experiment (Fig. 6A and B). Consistently, wild-type mice were able to recover from colitis, as shown by the gross morphology and histopathology of the colons at day 13 post-DSS (Fig. 6C and D). In contrast, Casr−/− mice still displayed severe colonic inflammation and mucosal damage (Fig. 6C and D), indicating that the intestinal CaSR plays a key role in the regulation of induced-inflammation.

4. Discussion

We demonstrate that CaSR is a key molecule expressed in gut epithelial cells that contributes to the preservation of intestinal epithelial cell integrity, and maintenance of immune homeostasis in the gut, the disruption of which results in intestinal
Fig. 5. (1.5 columns) Colonic T cell phenotype in Casr<sup>−/−</sup> mice. Cell surface expression of IL1R (A) and PD-1 (B) on CD4<sup>+</sup> and CD8<sup>+</sup> T cells was analyzed by flow cytometry. Data represent observations from two independent experiments and are shown as mean ± S.E.M. *P < 0.05 compared to WT. n = 5 mice/group.

Fig. 6. (1.5 columns) Casr<sup>−/−</sup> mice are highly susceptible to DSS-induced colitis. Casr<sup>−/−</sup> and Casr<sup>+/+</sup> littermate controls were given 3% DSS in the drinking water for 7 days. Disease progression was scored by weight loss (A), diarrhea (B), gross inflammation of the colon (C), and histopathology (D). Data are shown as mean ± S.E.M. *P < 0.05, **P < 0.01 compared to WT. n = 5 mice/group. 20× magnification.
inflammation. Recently, it was shown that "global" Casr/parathyroid hormone (Pth) double knockout mice had chronic intestinal inflammation, with increased neutrophil infiltration, myeloperoxidase activity, tumor necrosis factor receptor 1 (TNFR1) expression [46], and susceptibility to DSS [47]. However, given the global nature and multiple factors involved in that study, it was difficult to ascertain the cause of the inflammation. Nonetheless, the study suggested that intestinal CaSR may be anti-inflammatory. Indeed, activation of CaSR by its dietary agonists, calcium, spermine, or tryptophan, reduced inflammation (Tang & Cheng, unpublished observations), whereas inhibition of the receptor by depletion of dietary calcium enhanced gut inflammation in animal models of induced colitis [48]. Interestingly, while gut-specific CaSR detects nutrients and is potentially anti-inflammatory, the CaSR in murine bone marrow-derived macrophages/monocytes is pro-inflammatory in that it detects the "danger signal" (i.e., Ca released as a result of tissue injury) and activates the NLRP3 inflammasome [49]. Thus, role of CaSR in inflammation appears to be cell-type specific.

Nutrient availability and nutrient-sensing in the host significantly contribute to gut homeostasis and the immune responses induced [48,50–61]. In the present study, we demonstrate that a single deficiency in epithelial CaSR altered the composition of the gut microbiota, with Casr−/− mice having significantly increased Deferrribacteraceae and reduced abundance of lactobacilli and C. coccoides (cluster XIVa of the genus Clostridium), despite identical environmental conditions. Clostridium cluster XIVa species are beneficial commensal bacteria that induce butyrate production, Treg development, maintenance of barrier function, and competition with pathogens in gut colonization. The specific depletion of commensal Clostridia, together with the outgrowth of small groups such as Deferrribacteraceae, may thus contribute to the disruption of mucosal homeostasis and subsequent activation of local immune responses seen in Casr−/− mice. Indeed, depletion of Clostridia has been consistently associated with chronic intestinal inflammation in patients with IBD [62–65] and atopy [66], whereas Deferrribacteraceae was shown to correlate with the level of inflammatory responses in C. rodentium-infected mice [38]. Whether these changes are a cause or a consequence of inflammation, or consequent to the downregulation of the anti-inflammatory CaSR, requires further investigation.

The immune responses observed in Casr−/− mice suggest that intestinal epithelial CaSR deficiency leads to a shift in local and systemic innate and T cell immune responses from a status characterized by regulation to one that is highly stimulated. However, since the expression of PD1, a molecular signature of T cell exhaustion [67], was significantly enhanced on T lymphocytes, the activated immune phenotype displayed in the immune cells of Casr−/− mice may not necessarily indicate active, effective immunity, but evidence of chronic intestinal inflammation. In further support of chronic inflammatory responses, Th1 and Th17 responses were elevated in Casr−/− mice. Interestingly, FoxP3+ Tregs were not decreased, but were increased in Casr−/− mice, particularly in the MLNs. Nonetheless, the quality of these Tregs warrants further investigation, as studies have highlighted the plasticity of these cells toward a proinflammatory phenotype [68]. Therefore, considering the heightened state of inflammation in Casr−/− mice, it is conceivable that Casr−/− mice-derived FoxP3+ Tregs may be functionally defective. This state of chronic inflammation rendered Casr−/− mice highly susceptible to DSS-induced colitis, indicating that, in addition to defective intestinal barrier function and increased inflammation, CaSR deficiency may also result in impaired wound healing in the gut.

In summary, the present study demonstrates that attenuated CaSR signaling in the gut epithelium leads to enhanced permeability of the epithelial barrier, resulting in translocation and dissemination of luminal bacteria and activation of local and systemic innate and adaptive proinflammatory immune responses. The subsequent excessive gut inflammation disrupts the intestinal milieu and impairs gut homeostasis, thereby contributing to susceptibility to autoimmune/inflammatory diseases, such as IBD. Thus, the CaSR may serve as a potential therapeutic target for a range of autoimmune-inflammatory intestinal disorders, including IBD and colon cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.05.007.

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