Building Tolerance to Gut Bacteria: Quantification of Goblet Cell – Immune Interactions in the Proximal and Distal Colon

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Bachelor's Degree Project, MOBK01
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Spring 2021

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Abstract

Goblet cells, specialized mucus secreting cells lining the gastrointestinal lumen, are key players in mediating immune responses in the gut. Secreting the major component of the mucus layer; mucin 2, a barrier against the intrusion of foreign pathogens into the host is maintained while simultaneously sampling and delivering luminal substances to the immune system through the formation of goblet cell associated passages (GAPs). This process, called luminal surveillance, promotes the induction and maintenance of tolerance against dietary substances and innocuous commensal microbes and is crucial in the maintenance of homeostasis and gut health. The disruptions of goblet cell function leads to chronic inflammation seen in e.g. inflammatory bowel disease (IBD) composed of Crohn's disease and ulcerative colitis . To get a deeper understanding of the role goblet cells have in tolerance and disease and how their interactions are regulated, the quantity of interactions between goblet cells and immune cells in the proximal and distal colon was investigated and compared. Counter to my hypothesis, the proximal colon showed more frequent interactions in the crypts, than the crypts of the distal colon. Further studies need to be conducted on what factors facilitate the interaction between GCs and LP-APCs in the colon and what specific function the interaction have, as well as if this interaction induces oral tolerance.

Introduction

The epithelial lining of the gastrointestinal tract is a site for nutrient absorption of dietary antigen, immune surveillance and innate, adaptive and tolerogenic immune responses against foreign pathogens and commensal microbes. Commensal microbes degrade and convert polysaccharides to fatty acids thereby increasing the availability of nutrients to the host. In return they receive an ideal living environment, allowing them to occupy a niche which prevents the colonization of pathogens in the lumen (Knoop et al., 2018). Tolerance towards dietary antigens and the microbiota is crucial for homeostasis and gut health. Complex interactions between the immune system, epithelium and goblet cells enable simultaneous promotion of tolerance and responsiveness to invading pathogens by sampling of luminal content; luminal surveillance. Breakdown of these interactions lead to disruption of homeostasis and breakdown of tolerance, resulting in chronic inflammation seen in e.g. inflammatory bowel disease (IBD) composed of Crohn's disease and ulcerative colitis (Flannigan et al., 2015).

Goblet cells (GCs) are specialized epithelial cells, making up 16% of cells lining the gastrointestinal lumen, which produce and secrete mucin 2 (MUC2); the major component of the mucus layer which functions as a physical barrier separating the epithelial cells from the microbiota (Johansson et al., 2008). The importance of the mucus layer was demonstrated in a study by Van der Sluis et al. (2006) showing that lack of MUC2 protein and mucus secretion in mice lead to the development of spontaneous colitis. The mucus layer consists of an inner and outer layer. The microbiota resides in the outer looser layer and interacts with oligosaccharides of mucin glycoproteins which provide an attachment site and act as energy source, whereas the inner layer is clear of bacteria and serves as a buffer between epithelium and microbiota. In the colon this inner, firmer layer is attached to the epithelium, in contrast to the soluble mucus in the small intestine (Johansson et al., 2008; Kim & Ho, 2010; Knoop et al., 2018). Segmental differences in mucus barrier properties also exist along the colon due to differences in the thickness and permeability of the inner mucus layer. Proximal colon mucus is thinner and partly permeable, whereas distal colon mucus is thicker and impermeable to bacteria (Ermund et al, 2013; Johansson et al., 2008).

The mucus secreted by GCs is necessary in the defense against the commensal flora, intestinal pathogens, and the elimination of gut content, but GCs also aid in the induction and maintenance of tolerogenic immune responses by sampling the luminal content. GCs form goblet cell associated passages (GAPs) that deliver luminal antigens across the epithelium to the underlying immune cells, the antigen presenting cells (APCs) in the lamina propria (LP). This pathway of transepitelial antigen delivery to the immune system was shown to be the most common in steady state which points at the importance of GCs for immune surveillance (Knoop et al., 2015).

Regional differences of GAP formation along the intestine can be observed. GAPs are present in the small intestine and distal colon during steady state but not in the proximal colon due to inhibition by the microbiota via epidermal growth factor receptor (EGFR) pathways.
GAP formation is induced by acetylcholine (ACh) binding to the muscarinic ACh receptor (mAChR) 4 on the surface of GCs. In the presence of the microbiota, EGFR activates mitogen activated protein kinase (MAPK) which inhibits the ACh response (Knoop et al., 2017). The abundance of mucus but the absence of GAPs and tolerance induction in the proximal colon, suggests that GCs secrete mucus to maintain a barrier while simultaneously limiting GAP formation in the response to microbial sensing. The purpose ; minimize the exposure of luminal antigen to the immune system in the case of an unfavorable gut environment which could trigger an immune response (Knoop et al., 2015). How this process of maintaining a barrier while simultaneously exposing the immune system to antigen is balanced and regulated is still an unanswered question in mucosal biology.

Studies suggest another role for GCs in oral tolerance. Interactions between GCs, specifically MUC2, and APCs lead to an imprint of mucosal properties and the expression of an anti-inflammatory phenotype required for oral tolerance. LP-DCs acquire tolerogenic properties necessary for the induction and maintenance of $Foxp3^+$ T regulatory cells (pTregs) which play a crucial role in mediating specific tolerance. Furthermore, the interaction of GCs with LP macrophages induces cytokine IL-10 production. Keeping this in mind, it is feasible that the removal of GAPs promotes intestinal inflammatory diseases due to loss of tolerance (Knoop et al., 2018; Kulkarni et al. 2020).

Stromal cells have long been regarded as plain structure providing cells for lymphoid organs but have been discovered to express high levels of MHCII with the ability to sample antigen (Roozendaal & Mebius, 2011). If this subtype of APC interacts with GCs in the colon to drive tolerance is not known.

Multiple markers can be used to identify and distinguish between subpopulations of macrophages and DCs. The main identifier of LP-APCs is the major histocompatibility complex (MHC) II. Cell surface markers like F4/80 can distinguish between macrophages and DCs. Macrophages in the intestine can be identified using F4/80, CD11b, CX3CR1, CD64, CD14 and CD68. DCs express CD103 but lack CX3CR1 and can be divided into $CD11b^{+}$ and $^{-}$ subsets. DCs also express CD272 and CD26 (Flannigan et al., 2015). The CX3 chemokine receptor 1 (CX3CR1) ligand CX3CL1 is highly expressed in GCs, making CX3CR1 an interesting marker for the study of GC-$CXC3CR1^{+}$ APC interactions.

Research by Veenbergen et al. (2016) shows the segmental differences in tolerance induction between the small intestine and the colon. Food antigens are largely taken up in the small intestine, while microbial antigen is of the majority in the colon, due to the distally increasing number of bacteria which reaches 10^{11} bacteria per milliliter of luminal content (Johansson et al., 2008). Migratory DCs, consisting of $CD103^{+}$ $CD11b^{+/-}$ and $CD103^{-}$ $CD11b^{+}$ (the latter with intermediate CX3CR1 expression) subpopulations, actively transport antigen from the intestine to draining lymph nodes to induce proliferation and differentiation of naive T cells. During steady state DCs deliver antigens to draining lymph nodes for induction of regulatory T cells (Treg) responses that play an important role in maintaining immune tolerance. Mesenteric lymph nodes (MLN) are central in driving tolerance in the small intestine but the Veenbergen et al. (2016) study concluded that colonic tolerance is induced in the distal colon draining caudal and iliac lymph nodes (ILN) and not in the MLN. The systemic differences between small intestine and colonic tolerance induction, namely, the different nature of antigens, DC subpopulation and inductive site of tolerance, depend on underlying local regulatory mechanisms of antigen sampling and delivery by GCs which remain undiscovered.

Purpose. Considering the central role that GCs and the mucus layer have in induction and maintenance of tolerance and how disruptions of GC function causes disease, a deeper

understanding of how GCs regulate tolerance and antigen uptake, what subpopulations of LP-APCs interact and what specific function these GC-LP-APC interactions have needs to be further explored.

The main purpose of this study is to investigate whether the absence of antigen uptake by GCs in the proximal colon is also reflected in the absence of GC-LP-APC interactions. To address this question I will compare GC-LP-APC interactions in the proximal and distal colon and correlate my findings to what is known regarding GAP formation in the proximal and distal colon.

Hypothesis. The mucus layer in the proximal colon is not as thick as in the distal colon, enabling bacteria to be in closer proximity to the epithelium, resulting in the inhibition of GAP formation and sampling of luminal content. Since GAPs forming GCs interact with and deliver antigens to the immune system, the amount of sampling GCs is expected to correlate with the number of interactions between GCs and LP-APCs. Based on this, I hypothesize that the overall frequency of GC-LP-APC interactions is lower in the proximal colon as compared to the distal colon.

Materials and Methods

Animals. Mice are widely used for studies of gastrointestinal physiology and pathophysiology since they can be easily genetically manipulated. The Cre/lox system allows cell-type-specific gene expression. The mice used in this study have a Rosa26 knock-in, expressing tdTomato fluorescent protein in the cytoplasm of Muc2 expressing cells e.g. all goblet cells. Experiments were conducted on five Muc2Cre-RosadtRFP animals. Mice were housed in a specific pathogen-free facility, under controlled temperature (21–22 °C), humidity and 12-h light/dark cycle. Mice were euthanized by cervical dislocation under ketamine/xylazine anesthesia. All animal experiments were approved by the Swedish Laboratory Animal Ethical Committee in Gothenburg, Sweden and Jordbruksverket, Jönköping, Sweden.

Genotyping. Following the HotSHOT DNA extraction protocol to isolate DNA from ear biopsies using alkaline lysis and neutralizing reagent, 10 µl of final DNA solution was used in the PCR. An ethidium bromide agarose gel (50 µg EtBr/100 ml agarose) was cast for gel electrophoresis and samples were run at 120 V. By documenting the gel and comparing sample bands with the positive control, RFP^+ Cre^+ mice could be identified to be used in experiments for tissue extraction.

Tissue preparation. Colonic tissue was dissected, washed in PBS and fixed overnight in 4% formaldehyde at 4°C. The tissue was transferred to a 30% sucrose solution overnight or until tissue sank. The tissue was embedded in OCT and prepared for cryosectioning. 6 µm sections were cut using a cryostat and placed on Thermo Scientific Menzel-Gläser Superfrost Plus slides.

Optimization of fluorescently labeled MHCII, CD34, CX3CR1, Vimentin, F4/80 and PDGFR-α antibody staining protocols.

CD34, Vimentin and PDGFR-α are stromal cell markers. F4/80, CX3CR1 and MHCII are APC markers. Different primary and secondary antibody concentrations and different antigen retrieval methods; citric acid, EDTA and SDS, were tested in order to optimize the staining protocols. Antigen retrieval is used to break methylene bridges that are formed during fixation which mask antigenic sites, to free antigen so that antibodies can bind.

MHCII Immunostaining. The OCT was removed by washing in PBS for 5 minutes and the sections were permeabilized in a 0.1% Triton solution for 5 minutes which was removed with a 5 min PBS wash. Sections were blocked by incubating in a 1% BSA solution for 30 minutes at room temperature. A rat anti-mouse MHCII monoclonal primary antibody (1:100; Invitrogen) and Alexa Fluor 647 goat anti-rat IgG secondary antibody (1:500, Invitrogen) were used. DNA was stained using Hoechst solution (2 μg/ml). An upright LSM 700 Axio Examiner. Z1 laser scanning confocal microscope was used to acquire images using the ZEN 2012 software.

Image analysis. Goblet cell and goblet cell interactions in the epithelial layer and crypts were counted by analyzing 10 images per section and animal, with a minimum of 40 counted crypts using Imaris software (Bitplane).

Statistical analysis. A two tailed, paired t-test was conducted to test the hypothesis.

Results

The optimization of different staining protocols for different antibodies proved necessary as CX3CR1, Vimentin, F4/80 and PDGFR-α staining resulted in high background fluorescence possibly resulting from nonspecific binding due to e.g. insufficient blocking. An antigen retrieval step was integrated into the protocol to revive the epitopes, but was not compatible with RFP which was demonstrated to be heat sensitive, resulting in loss of RFP signal during heat assisted antigen retrieval. Lowering the concentrations of the secondary antibody to minimize nonspecific binding gave no positive result.

Staining using fluorescently labeled MHCII and CD34 antibodies showed with some optimization of fixation time and antibody concentration sufficient RFP and MHCII signal. A micrograph of a distal colon section of Muc2Cre-RosadtRFP tissue stained with Alexa Fluor 488 labeled CD34 antibody (green) can be seen in Fig. 1.

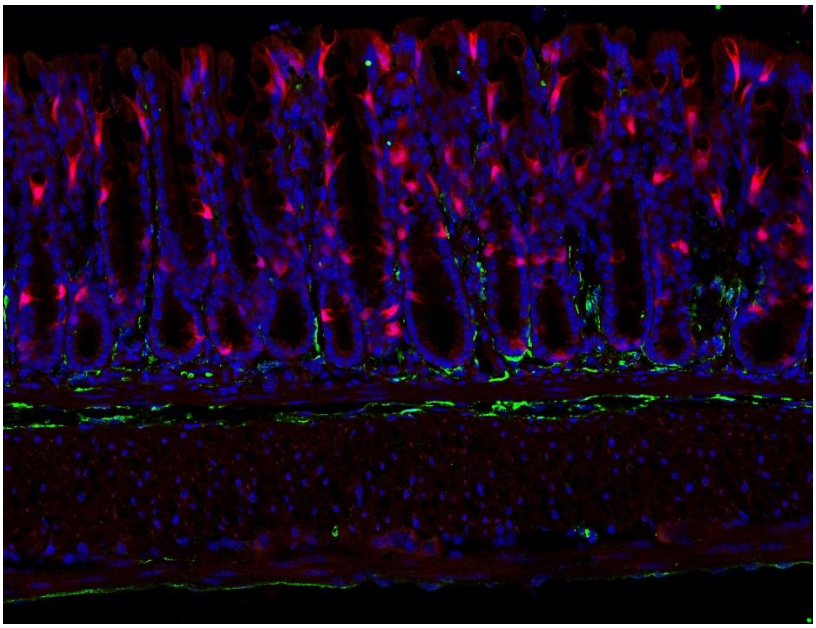

Figure 1. Immunostained distal colon section from Muc2Cre-RosadtRFP mouse, depicting $CD34^+$ APCs (green), RFP^+ GCs (red), DNA (blue).

Focusing on immunostainings using MHC II antibody, GC-LP-APC interactions could be quantified (Fig. 2b).

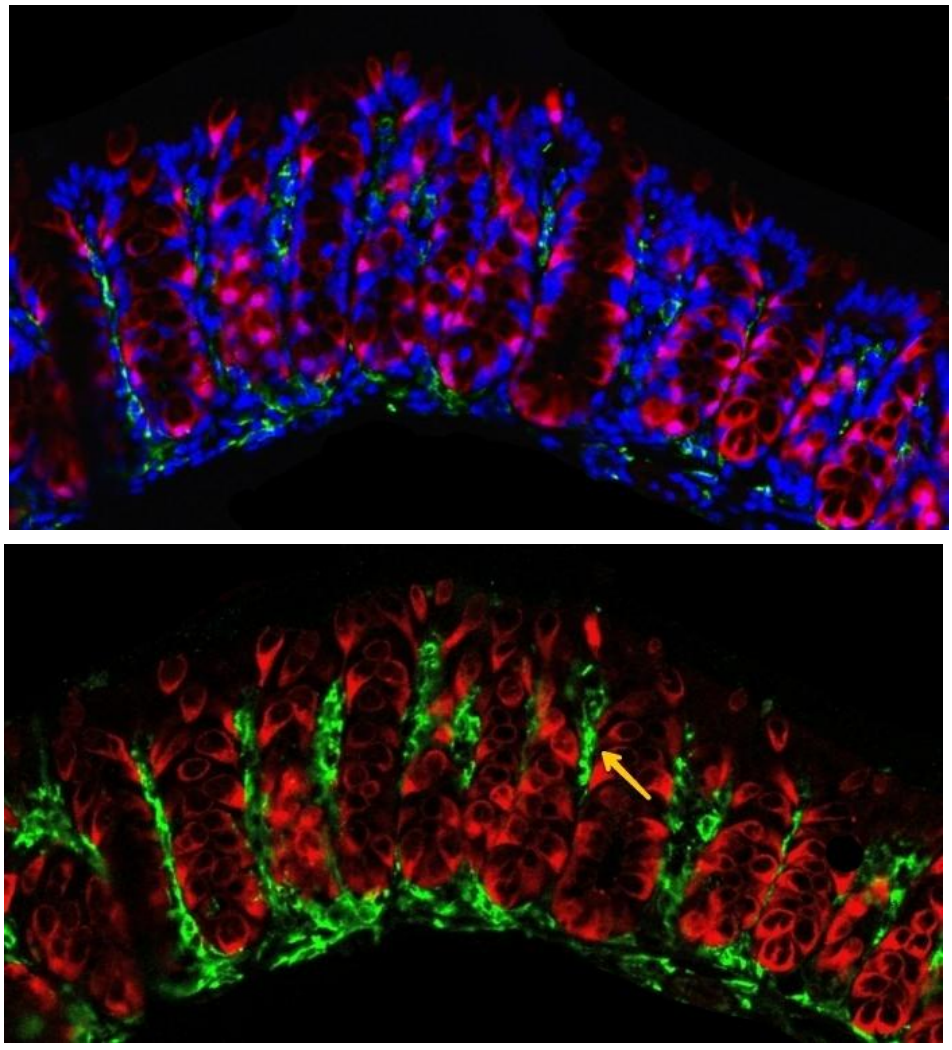

Figure 2 a. Immunostained proximal colon section from Muc2Cre-RosadtRFP mouse, depicting MHCII^{+} APCs (green), RFP^{+} GCs (red), DNA (blue).
b. Proximal colon section without the DNA stain. The yellow arrow points at a GC (red) -APC (green) interaction.

Micrographs of immunofluorescent stained proximal colon sections in Fig. 2 a/b depict shorter crypts as compared to distal colon crypts (Fig. 3).

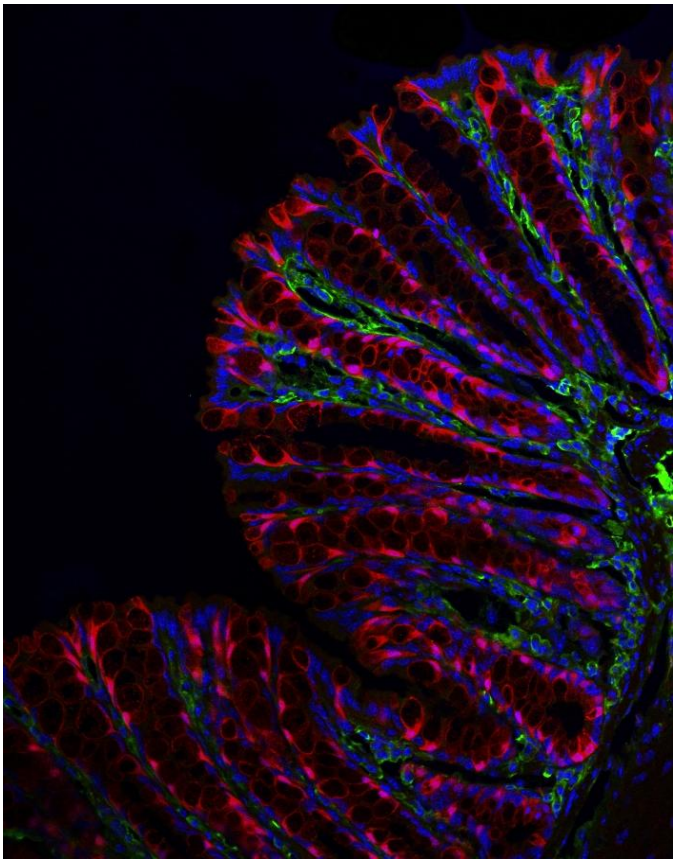

Figure 3. Immunostained distal colon section depicting MHCII^{+} APCs (green), RFP^{+} GCs (red), DNA (blue).

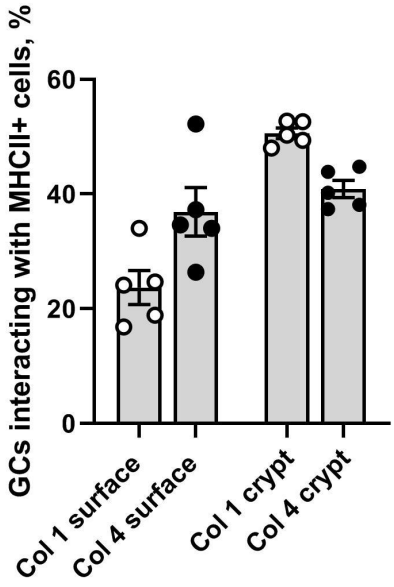

Figure 4. Percentage of GCs interacting with MHCII^{+} cells in the epithelial layer and crypts of the proximal colon (Col 1) and distal colon (Col 4). Each data point represents an individual mouse.

The percentage of GC-($MHCII^+$) APC interactions are approximately 30% in the surface epithelium and 45.7% in the crypts across both sections (Fig. 4). The number of GC-LP-APC interactions in the surface epithelium of the proximal colon ranges from 16.8 to 34.0 %, with a mean of 23.7 %. The number of GC-LP-APC interactions in the surface epithelium of the distal colon ranges from 26.4 to 52.2 % with a mean of 36.9%. Summarizing, surface GCs in the proximal colon seemingly have less interactions with LP-APCs than surface GCs in the distal colon (13.2% mean decrease). This difference is not statistically significant due to the spread in data points ($t=1.922$, $p > 0.05$). Although there is no statistically significant difference between segments, a clear trend can be seen.

The number of GC-LP-APC interactions in the crypts was significantly higher in the proximal colon (mean of 50.2%) compared to the distal colon (mean 40.9%) ($t=5.947$, $p < 0.05$).
The spread of the amount of GC interactions in the crypts among the animals was relatively small compared to the surface epithelium data, consequently showing a statistically significant difference between segments. The amount of interactions in the surface epithelium compared to the crypts differed less in the distal colon (mean difference = 4%), compared to surface epithelium and crypt interactions in proximal colon (mean difference = 26.9%).

Discussion

The results from this study suggest less frequent GC-LP-APC interactions in the surface epithelium of the proximal colon compared to distal colon, corresponding with my hypothesis. Contradicting my hypothesis, GC-LP-APC interactions in the proximal crypts could be observed more frequently than in the distal colon. I expected bacterial inhibition of GAP formation in the proximal colon to also inhibit GC-LP-APC interaction. While this was the case in the surface epithelium of the proximal colon, the crypts of the proximal colon showed significantly more interactions than the crypts of the distal colon.

An explanation for why this could be, is although the mucus layer covering the proximal colon is thinner, this mainly results in direct interactions between bacteria and the surface epithelium. Bacteria do not frequently enter the proximal colon crypts, and consequently, interactions between crypt GCs and LP-APCs remain uninhibited. The interaction frequency in the surface epithelium and crypts being notably similar in the distal colon suggests that the thicker mucus layer promotes GC-LP-APC interactions at both sites. Possibly these interactions are caused by luminal sampling and mediate tolerance induction.

Studies using antibiotics, and dextran sodium sulphate (DSS) treatment to disrupt microbiota and the mucus layer, respectively could be conducted to investigate whether the observed differences in GC-LP-APC interactions are due to differential exposure to the microbiota. Previous studies reveal that the translocation of commensal microbes following antibiotic treatment was associated with the formation of GAPs in the colon, alluding to the inhibition of GAP formation by bacteria (Knoop et al., 2017). The question remains to be answered whether antibiotic treatment would have an impact on the number of GC-LP-APC interactions, specifically if GC-LP-APC interaction frequency in the proximal colon could be increased. Dextran sodium sulphate (DSS) destroys the inner, usually impermeable mucus layer, making it possible for bacteria to penetrate, inducing inflammatory responses against commensal bacteria (Johansson et al., 2010). This model of inducing colitis could be used to destroy the mucus layer and test if increasing direct contact between surface GCs and the microbiota would inhibit GC-LP-APC interactions in the distal colon.

Further studies need to be conducted on what factors facilitate the interaction between GCs and LP-APCs in the colon and what specific function the interaction have, as well as if this interaction induces oral tolerance. MHCII is a very broad APC marker and further studies are needed that focus on phenotyping specific subpopulations that interact with GCs at the different sites to determine what physiological role these interactions have in maintaining gut homeostasis.

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