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On cell death in the intestinal epithelium and its impact on gut homeostasis

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Abstract

PURPOSE OF REVIEW: Both apoptotic and non-apoptotic cell extrusion preserve the barrier functions of epithelia. Live cell extrusion is the paradigm for homeostatic renewal of intestinal epithelial cells (IEC). By extension, because extruded cells are not apoptotic, this form of cell shedding is thought to be largely ignored by lamina propria phagocytes and without immune consequence.

RECENT FINDINGS: Visualization of apoptotic IEC inside distinct subsets of intestinal phagocytes during homeostasis has highlighted apoptosis as a normal component of the natural turnover of the intestinal epithelium. Analysis of phagocytes with or without apoptotic IEC corpses has shown how apoptotic IEC constrain inflammatory pathways within phagocytes and induce immunosuppressive regulatory CD4 T cell differentiation. Many of the genes involved overlap with susceptibility genes for inflammatory bowel disease (IBD).

SUMMARY: Excessive IEC death and loss of barrier function is characteristic of IBD. Because regulatory and tolerogenic mechanisms are broken in IBD, a molecular understanding of the precise triggers and modes of IEC death as well as their consequences on intestinal inflammation is necessary. This characterization should guide new therapies that restore homeostatic apoptosis, along with its associated programs of immune tolerance and immunosuppression, to achieve mucosal healing and long-term remission.

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Conflict of interest statement

None

Keywords

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Introduction

A convergence of genetic susceptibility factors, the enteric microbiota and injury to the intestinal epithelium underlies the pathogenesis of inflammatory bowel disease (IBD) (1, 2). Damage of the intestinal epithelium manifests in a notable increase in the death of intestinal epithelial cells (IEC) (3–9). High levels of cell death termed ‘apoptosis’ were noted in the epithelium of patients with ulcerative colitis (9–11). An association between apoptosis and IBD has also been found in epithelial cell proteome studies in both IBD patients and IBD mouse models (12, 13). IEC necroptosis is thought to underlie the microerosions and epithelial gaps observed in mice and humans by *in vivo* imaging (14, 15), and may serve as a pathophysiological mechanism that precipitates barrier dysfunction leading to inflammation. In all these cases, the most obvious effect of increased IEC death is disruption of the IEC barrier and consequent loss of its protective and antimicrobial activities leading to dysbiosis and microbial translocation into the sterile intestinal lamina propria. These events drive further inflammation and more damage to the intestinal epithelium, making it difficult to distinguish cause from effect. This review examines the different modes of cell death that have been reported in the intestinal epithelium and the conditions under which they occur. It also highlights apoptosis as the physiological form of cell death that can occur during intestinal epithelial turnover. The consequences of innate recognition of apoptotic IEC on intestinal tolerance and homeostasis are discussed and their relevance to IBD.

Apoptosis during homeostatic turnover of the intestinal epithelium

Apoptosis is the preferred mode of cell death during both embryonic development and adult tissue turnover, and its balance with cell division maintains proper tissue size and function (16). Within the intestine, continuous turnover of the intestinal epithelium is critical for ensuring effective barrier function against digested food and the commensal microbiota. IEC arising from stem cells at the base of the crypts travel towards the villi tips in the small intestine or luminal face of the crypts in the large intestine (17). This process takes 4–5 days at the end of which IEC are shed into the lumen through mechanisms debated to involve either apoptosis or live extrusion by upwardly moving cells (18). Basolateral contraction of actin and myosin during IEC extrusion precedes the appearance of the characteristic readouts of apoptosis, caspase-3 cleavage and phosphatidylserine exposure (19, 20). Thus, a commitment to apoptosis can signal extrusion of IEC that have not yet exhibited the hallmarks of apoptosis.

On the other hand, IEC-specific deletion of caspase-8, a critical orchestrator of apoptosis, did not lead to discernible abnormalities in the intestinal epithelium in mice, thereby undermining the role of apoptosis in the cycle of epithelium turnover (8, 21). Caspase-8-deficiency led to IEC death by inflammatory necroptosis and precipitated spontaneous terminal ileitis, Paneth cell loss, and high susceptibility of mice to dextran sulfate sodium

(DSS)-induced colitis (21). Similarly, IEC-specific deletion of FAS-associated death domain protein (FADD), an adaptor that conveys signals from tumor necrosis factor (TNF) receptor 1 (TNFR1) or FAS to caspase-8, leads to spontaneous IEC necroptosis with loss of Paneth cells and both small and large intestinal inflammation (22). Disruption of the non-inflammatory process of apoptosis drove necroptosis concomitant with increased expression of the central kinase for necroptosis, receptor-interacting serine/threonine protein kinase 3 (RIPK3) whose levels were increased in the terminal ileum of patients with Crohn's disease (21). While live IEC extrusion is likely unaffected by caspase-8 or FADD deficiency, the findings demonstrate a commitment to death at least in some IEC, either as an end to a short lifespan or in response to a specific signal. IEC destined to die will undergo death and if not by apoptosis then by necroptosis. By extension, if such a commitment to death is made under homeostatic conditions, it stands to reason that the favored mode of cell death would be non-inflammatory apoptosis.

Apoptosis and necroptosis in intestinal inflammation

Unlike programmed apoptosis that preserves tissue function, excessive apoptosis in the intestinal epithelium compromises barrier integrity and leads to inflammation (18, 23). TNF-α, a critical molecule and therapeutic target in IBD (24), induces excessive IEC shedding (25, 26). Systemic and intestinal tissue levels of TNF-α are increased in IBD patients (27), and genome wide association studies (GWAS)-identified IBD risk alleles associated with TNF signaling (*RELA*, *NFKB1*, *TNFAIP3*), point to a critical role for TNF-α in IBD pathogenesis (28). Upon binding TNFR1, TNF-α can induce apoptosis upon deubiquitylation of the receptor-interacting serine/threonine protein kinase 1 (RIPK1) by ubiquitin modifying proteins such as A20 (*TNFAIP3*), but it can also induce necroptosis upon caspase-8 or A20 inhibition and the heterodimerization of RIPK1 with RIPK3 (29–31). TNF-α-induced IEC shedding is reported to be associated either with IEC apoptosis (14, 32), or with necroptosis where multiple IEC are shed and barrier integrity is lost driving inflammation (15, 26). Excessive TNF-α-induced IEC shedding is thus unlike the homeostatic IEC extrusion where rapid redistribution of tight junction proteins and replacement of extruded cells is observed (33, 34). Replacement of homeostatic IEC apoptosis with pathogenic inflammatory cell death may play a critical role in IBD pathogenesis (Figure 1).

Despite the central role of RIPK1 in mediating apoptosis or necroptosis downstream of TNF-α/TNFR1 signaling, IEC-specific deletion of RIPK1 led to severe intestinal inflammation associated with IEC apoptosis mediated in part by TNFR1 but also by interferon and TLR signaling (35, 36). This unexpected phenotype revealed a scaffold function of RIPK1 that signals cell survival by preventing the degradation of pro-survival proteins, and is independent of its kinase activity that promotes apoptosis and necroptosis (31, 35–37). Indeed, mice harboring a mutant RIPK1 lacking kinase activity (kinase-dead) did not exhibit intestinal inflammation (36). These findings uncovered a different facet of RIPK1 that preserves rather than compromises IEC survival through poorly understood, kinase-independent scaffold functions. In fact, concomitant deletion of RIPK1 from FADD or caspase-8 deleted IEC (see above), rescued IEC and abolished the inflammatory phenotype in mice (35, 36). Interestingly, although RIPK3-mediated necroptosis was the

culprit in the pathology of FADD or caspase-8 IEC deleted mice (21, 22), its concomitant deletion in FADD or caspase-8 deleted IEC had no effect on IEC death, intestinal pathology or inflammation demonstrating the unique role that RIPK1 plays in protecting the intestinal epithelium (35, 36).

Recent evidence shows that A20 and its binding partner ABIN-1 (*TNIP-1*) – single nucleotide polymorphisms in which correlate with susceptibility and severity of IBD (28, 38) – play a dual IEC-intrinsic role in protecting the intestinal epithelium from RIPK1-mediated apoptosis and necroptosis (39). Simultaneous IEC-specific deletion of A20 and ABIN-1 leads to IEC apoptosis, severe spontaneous enterocolitis, and mouse lethality (39). IEC-derived TNF-α was a prominent mediator, and A20 and ABIN-1 inhibited TNF-induced caspase-8 activation and RIPK1 activity. Neutralization of IEC-derived TNF-α is perhaps one mechanism underlying the decreased apoptosis and mucosal healing reported after anti-TNF therapy (40, 41). TNF-independent, MyD88-dependent mechanisms, functioning within the hematopoietic cell compartment, also mediated IEC apoptosis and appeared to be particularly controlled by ABIN-1 (39). Identification of these mechanisms could provide additional therapeutic targets for IBD patients who fail anti-TNF therapy. Collectively, the findings also point to the merit of identifying IBD patients with lower mucosal tissue levels of A20 and/or ABIN-1 (42–45), as these patients might particularly benefit from RIPK1 inhibitors presently in clinical trials on subjects with active ulcerative colitis clinical trials ((46, 47) and clinicaltrials.gov/ct2/show/NCT02903966). Reduced A20 expression has also been reported in monogenic early onset inflammatory diseases characterized by mucosal ulceration (48).

Apoptotic intestinal epithelial cells are sampled by intestinal lamina propria phagocytes

In 2000, apoptotic intestinal epithelial cell (IEC) DNA and cellular remnants were reported inside a distinct subset of rat intestinal lymph-borne dendritic cells (DC) originating from the lamina propria and Peyer's patches and migrating to T cell areas of the Peyer's patches and mesenteric lymph nodes (MLN) (49). Apoptotic IEC sampling by DC was also noted in the lamina propria and MLN of gnotobiotic rats suggesting that transport of apoptotic IEC to MLN was constitutive and independent of the presence of the microbiota (49). In mice, transgenic expression of the model antigen ovalbumin (OVA) specifically within the intestinal epithelium demonstrated the ability to induce tolerance of adoptively transferred OVA-specific CD8 T cells and precipitate intestinal tissue damage during infection with an OVA-expressing virus (50). These observations lend support to DC-dependent transport of apoptotic IEC antigens to the MLN for the induction of tolerance or immunity to epithelium derived antigens. In 2004, murine DC within the subepithelial dome underlying the Peyer's patch follicle-associated epithelium were found to contain apoptotic epithelial cells both at steady state and during reovirus infection – where reovirus antigen from virus-infected apoptotic IEC was presented to CD4 T cells (51).

More than a decade later, development of a transgenic mouse model expressing green fluorescent protein (GFP) specifically within IEC enabled the tracking of apoptotic IEC-

derived GFP into the complex network of mononuclear phagocytes present within the lamina propria of the small intestine (52, 53). These phagocytes sample luminal commensals and pathogens alike and orchestrate tolerance or the appropriate effector immune response (52, 53). Phagocytes within tissues can also internalize apoptotic tissue-derived cells (54). GFP-labeled apoptotic IEC were tracked into two subsets of intestinal lamina propria $CD64^+$ macrophages, $CD103^-CD11b^+$ and $CD103^+CD11b^+$, as well as a distinct subset of $CD24^+CD103^+CD11b^-$ DC that migrated to the MLN and instructed the differentiation of regulatory CD4 T (T_{REG}) cells (55). These findings revealed two important facets of the intestinal epithelium: (1) non-inflammatory apoptosis is a component of the natural life cycle of an IEC, and (2) apoptotic IEC are sampled by intestinal mononuclear phagocytes at steady state and homeostatic conditions.

Apoptotic IEC sampling orchestrates a broad program of immunosuppression

Visualization of the apoptotic IEC-derived GFP within intestinal lamina propria phagocytes enabled the isolation of phagocytes that carried or did not carry apoptotic IEC and a comparison of their transcriptional profile (55). Apoptotic IEC sampling was associated with a suppression of inflammation signature within engulfing phagocytes, although the specific genes and pathways involved varied between DC and macrophage reflecting specialized responses to apoptotic IEC. Interestingly, apoptotic IEC sampling was associated in all three phagocytes with a downmodulation of *Itgb7* encoding integrin-β7 that mediates homing to gut-associated lymphoid tissue and serves as the target for IBD therapy with Vedolizumab and Etrolizumab (56). Intestinal lamina propria $CD103^+CD11b^+$ and $CD103^-CD11b^+$ macrophages that carried apoptotic IEC downmodulated genes encoding pattern recognition receptors such as *Tlr2* and the C-type lectin receptors *Clec4a*, *Clec4b1*, *Cd209a* (55). These macrophages also downregulated *Alox5ap* encoding arachidonate 5-lipooxygenase (55), which facilitates inflammatory leukotriene biosynthesis (57). $CD103^+$ DC, on the other hand, downregulated transcripts related to components of the inflammasome including *Nlrp3*, *Casp1*, and *Il1a*, as well as genes encoding MAPK/ERK signaling proteins including *Lrrk2*, *Map3k4*, and *Fos*.

Notably, several of the differentially expressed genes between apoptotic IEC bearing and non-bearing phagocytes overlapped with susceptibility genes associated with IBD (28). Leucine rich repeat kinase 2 (LRRK2), a multifunctional protein kinase known to be a prominent contributor to familial Parkinson's disease (58), is a susceptibility gene for Crohn's disease (28). LRRK2 transcript levels were increased in inflamed compared to non-inflamed biopsies from Crohn's patients and the *LRRK2* M2397T risk allele for Crohn's disease impaired LRRK2 expression levels (59). *LRRK2* M2397T was significantly associated with Paneth cell defects in Japanese Crohn's disease patients instead of the autophagy gene *ATG16L1* T300A polymorphism in North American Crohn's disease (60). *IL12b* encoding the p40 subunit shared by IL-12 and IL-23 was also upregulated in apoptotic IEC bearing intestinal lamina propria $CD103^+$ DC (55), and single nucleotide polymorphisms in *IL12b* are associated with Crohn's disease in Korean populations (61)

A prominent transcriptional signature associated uniquely with the CD103$^+$ DC that had sampled apoptotic IEC was the induction of negative regulators of inflammatory signaling (55). Within this category was *Tnfaip3* encoding A20 (55). A20 inhibits NF-κB signaling (38), RIG-I signaling (62), and NLRP3 inflammasome activation (63, 64). Enhanced apoptotic cell engulfment, presentation of apoptotic cell antigens, and activation of self-reactive T cells has also been associated with A20 deficiency in DC (38). DC induction of A20 in response to apoptotic cells may thus serve not only to inhibit intestinal inflammation, but also mediate tolerance to IEC. Deletion of A20 in DC leads to the development of pathologies in mice similar to those seen in humans with IBD and systemic lupus erythematosus (SLE) including colitis, ankylosing spondylitis, autoantibody development, nephritis, and splenomegaly (38). Total deficiency in A20 in mice leads to severe systemic inflammation, cachexia, increased sensitivity to lipopolysaccharide and TNF-α, and premature death (38).

Apoptotic IEC sampling mediates immune tolerance and induction of regulatory CD4 T cells

A distinct transcriptional signature uniquely associated with the apoptotic IEC carrying CD103$^+$ DC was the activation of regulatory CD4 T (T_{REG}) cells expressing the transcription factor Foxp3 (55). These CD103$^+$ DC upregulate *Aldh1a2* (55), which catalyzes the conversion of retinal to retinoic acid to generate T_{REG} cells in the intestine (65). Also upregulated is *Lrrc32* (55), which encodes LRRC32 (also known as GARP) associated with IBD susceptibility (66). The expression of GARP by DC in response to apoptotic IEC sampling may serve a role similar to that in T_{REG} cells where LRRC32 associates with and retains latent TGF-β on the cell surface to promote T_{REG}-mediated suppression (67).

Small intestinal lamina propria CD103$^+$ DC that had sampled apoptotic IEC expressed the highest levels of the chemokine receptor CCR7 that mediates migration to lymph nodes (55). These cells upregulated the suppressive co-stimulatory molecule *Cd274* (Programmed death ligand PD-L1) (55), which synergizes with TGF-β to promote the instruction of T_{REG} cell differentiation by DC, and whose expression on T_{REG} cells has been reported to promote their differentiation and maintain their function by enhancing Foxp3 expression (68). Also upregulated by these CD103$^+$ DC in response to apoptotic IEC sampling are the chemokine encoding genes *Ccl22* and *Ccl17* that have been found to induce efficient chemotaxis of intestinal lamina propria T_{REG} cells (69). Interestingly, CCL22 and CCL17 were reported to be selectively expressed by small intestinal lamina propria DCs at high levels, and that T_{REG} cells were in close proximity to these DCs (69). The collective upregulation of these genes upon apoptotic IEC sampling by lamina propria CD103$^+$ DC sets the stage for instructing differentiation of T_{REG} cells (55), enabling DC to directly impact the generation of these critical mediators of intestinal tolerance and homeostasis.

Migratory CD103$^+$ DC containing GFP-labeled apoptotic IEC were also detected at steady state within the MLN. These migratory DC shared a transcriptional profile with those carrying apoptotic IEC in the lamina propria (55). Several transcripts involved in T_{REG} cell

differentiation (*Cd274*, *Cd40*, *Aldh1a2*, *Ccl22*) were additionally increased in migratory MLN CD103$^+$ DC relative to those that did not contain apoptotic IEC, as well as transcripts encoding indoleamine 2,3 dioxygenase-1 (IDO-1) and IL-10 (55). IDO-1 promotes immune tolerance through the suppression of effector T cell responses and inflammatory cytokine production by DC, as well as the induction of T_{REG} cell differentiation (70), whereas IL-10 is a critical player in the induction of T_{REG} cells by tolerogenic DC (71). Apoptotic IEC carrying CD103$^+$ DC within the MLN were superior to their counterparts that did not contain apoptotic cells in the ability to induce Foxp3$^+$ T_{REG} cells *ex-vivo*, and notably without added TGF-β (55).

Conclusion

The natural cycle of IEC turnover by apoptosis has emerged as a critical component of the wide spectrum of innate, adaptive and microbiota dependent mechanisms that regulate intestinal homeostasis (52, 53, 72–74). GWAS have associated susceptibility to IBD with single nucleotide polymorphisms in genes involved in many of these pathways (28). Forty-five of the genes modulated in intestinal mononuclear phagocytes in response to the sampling of apoptotic IEC overlap with IBD susceptibility loci, the majority of which are in CD103$^+$ DC (55). How allelic polymorphisms within these loci affect genes involved in intestinal homeostasis and tolerance is poorly understood. A molecular delineation of cell death within the intestinal epithelium during healthy and disease states is necessary. Excessive TNF-α mediated apoptosis and necroptosis is expected to undo the homeostatic programs directed by apoptotic IEC sampling at steady state. Consistent with this prediction, the induction of intestinal epithelial ulceration in mice reversed the induction of T_{REG} cell activation genes in migratory MLN CD103$^+$ DC, pointing to potential disruption of tolerogenic apoptotic IEC sampling during IBD (55). It will be important to examine whether mucosal healing in response anti-TNF therapy restores these homeostatic programs to achieve long-term clinical remission in patients with IBD. A broader understanding of the integration between apoptosis within the intestinal epithelium and other mediators of intestinal homeostasis is a necessary step towards designing successful therapies for IBD.

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•• Of outstanding interest

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Key points

- Intestinal epithelial cells can undergo homeostatic apoptosis during natural turnover
- Apoptotic intestinal epithelial cells are sampled by lamina propria mononuclear phagocytes
- $CD103^+$ DC carrying apoptotic intestinal epithelial cells migrate to the mesenteric lymph nodes
- Apoptotic intestinal epithelial cell sampling mediates innate and adaptive immunosuppression

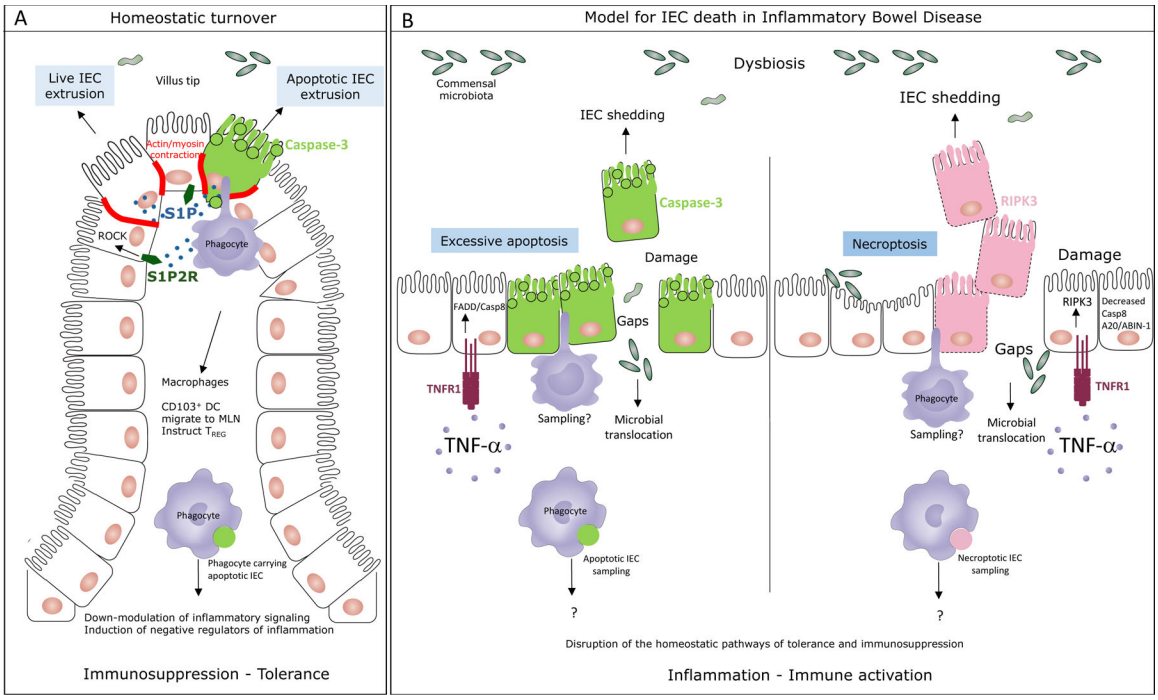

Figure 1. The mode of cell death in the intestinal epithelium impacts intestinal homeostasis.

A. Under homeostatic conditions, the intestinal epithelium undergoes natural turnover by continuous replacement of intestinal epithelial cells (IEC). Experimental evidence supports both apoptotic and non-apoptotic IEC extrusion at locations of overcrowding at the villi tips of the small intestine or luminal face of the crypts in the colon. Both of these processes require sphingosine 1-phosphate (S1P) signaling through S1P2 receptor (S1P2R) and resultant Rho associated kinase (ROCK) dependent actin/myosin contraction. ROCK can be cleaved and activated by caspase-3 during apoptosis to phosphorylate myosin light chain and induce apoptotic membrane blebbing. MHC class II^{+} CD11c^{+} intestinal lamina propria phagocytes, either macrophages or CD103^{+}CD11b^{-} dendritic cells (DC) sample the apoptotic IEC prior to their exclusion and respond by executing a broad program of immunosuppression. Apoptotic IEC carrying CD103^{+} DC also express negative regulators of inflammation such as the IBD susceptibility gene *TNFAIP3* encoding A20. These cells express the lymph node homing receptor CCR7 and induce the emergence of regulatory CD4 T (T_{REG}) cells. **B.** A proposed model for the types of IEC death that might occur during in inflammatory bowel disease. Increased levels of TNF-α in the intestinal lamina propria of patients with IBD induces IEC shedding that can be a result of excessive and unregulated apoptosis mediated by FADD and caspase-8 downstream of TNF receptor (TNFR1) engagement on IEC. Based on studies in mouse models, under conditions when caspase-8 or A20/ABIN-1 activity is impaired, TNF-α signaling through TNFR1 induces necroptosis mediated by the receptor interacting kinase 3 (RIPK3). Dying IEC are likely samples by intestinal phagocytes although formal proof of this process and its consequences is yet to come. In the absence of homeostatic apoptosis, it is expected that the immunosuppression and tolerance program in phagocytes, imparted by apoptotic IEC sampling, would be disrupted. Necroptosis is inflammatory in nature and would be expected to lead to the activation of intestinal phagocytes. Excessive apoptosis and necroptosis would

compromise barrier integrity and lead to the translocation of the commensal microbiota into the sterile lamina propria leading to more inflammation.