Morbus Crohn—a disease of failing macroautophagy in the immune system?

Sonja Meixlsperger and **Christian Münz**

Viral Immunobiology, Institute of Experimental Immunology, University Hospital of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Abstract

Mutations in genes involved in macroautophagy have been found to be associated with Morbus Crohn, also called Crohn's disease (CD), an inflammatory bowel disease. Taking this disease as an example for pathogenesis due to altered macroautophagy, we discuss here how macroautophagy supports innate and adaptive immunity. This support ranges from maintenance of components of the immune system, antigen processing for presentation to the immune system, to education of the immune system in order to distinguish self from dangerous non-self. A better understanding of these mechanisms should allow us not only to develop therapeutical strategies for CD but also to utilize macroautophagy for enhanced immunity against pathogens and tumors.

Introduction

Autophagy was first described as starvation-induced pathways in yeast that allow the cell to degrade cytoplasmic components, including whole organelles (1). Since the 1990s, the key autophagy machinery and its mammalian counterparts were identified as detailed in the article by Tamotsu Yoshimori in this issue. The most commonly studied of these pathways, on which we will also focus in this review, is called macroautophagy.

During macroautophagy, sheets of double membranes engulf part of the cytoplasm. On the molecular level, this requires two ubiquitin-like conjugation systems. The first one consists of a complex, encoded in yeast by autophagy-related gene 5 (*atg5*), *atg12* and Atg16-like 1 gene (*atg16L1*); among these, Atg12 is the ubiquitin-like molecule. This complex facilitates the lipidation of the second ubiquitin-like molecule, Atg8, for which at least six mammalian homologues exist in higher eukaryotes like humans. These are microtubule-associated protein 1 light chains A–C (MAP1LC3A, MAP1LC3B and MAP1LC3C), γ-aminobutyric acid (GABA) receptor-associated protein (*GABARAP*), GABARAP-like protein 1 [*GABARAPL1*; also known as glandular epithelial cell 1 [*gec1*] or Apg8/Atg8-like (*APG8L*)] and GABARAP-like protein 2 [*GABARAPL2*; also known as Golgi-associated ATPase enhancer of 16 kDa (*GATE-16*)].

Once coupled to its lipid ligand phosphatidylethanolamine, Atg8 incorporates into the autophagic membrane and mediates membrane fusion (2) until the isolation membrane is elongated enough to close to the fully mature autophagosome. The Atg5–Atg12–Atg16L1 complex and Atg8 on the outer membrane of this double-membrane-surrounded vesicle are recycled, whereas the Atg8 on the inner membrane stays luminal with it. These vesicles then fuse with late endosomes or lysosomes to form amphisomes or autolysosomes, respectively. Subsequently, hydrolases degrade the autophagosome content for recycling of nutrients and building blocks for macromolecules.

In addition to maintaining cellular homeostasis during times of starvation or stress, autophagy also plays a role in processes like aging, cancer, neurodegenerative disease and several aspects of innate and adaptive immunity (3, 4). Moreover, genome-wide association studies of inflammatory bowel disease (IBD) identified several mutations of autophagy associated genes to be associated with Crohn's disease (CD) (5, 6). These mutations affect *atg16L1* (7, 8), immunity-related GTPase family M (*IRGM*) (9, 10) and nucleotide-binding oligomerization domain containing 2 [*NOD2*, also known as caspase recruitment domain 15 (*CARD15*)] (11, 12), and they are exclusively associated with CD and not other forms of IBD.

First, the most prominent mutation in *atg16L1* that is associated with CD is *atg16L1T300A*. The mutation seems to decrease protein stability and possibly its interaction with the Atg5–Atg12 complex (13).

Second, mouse *Irgm*, but not human *IRGM* transcription, is up-regulated by IFN gamma (IFN-γ) and in turn mediates

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IFN-γ-dependent resistance to intracellular pathogens (14). For *IRGM*, single nucleotide polymorphisms associated with CD lie upstream of the gene and result in reduced expression levels of *IRGM*. Whereas over-expression of IRGM efficiently up-regulates macroautophagy in macrophages (15), reduced levels of IRGM lead to inefficient anti-bacterial macroautophagy (9).

Finally, NOD2 is the pathogen-associated molecular pattern (PAMP) receptor for muramyl dipeptide, a component of bacterial peptidoglycan. Ligand binding activates the nuclear factor κB and mitogen-activated protein kinase pathway resulting in the expression of pro-inflammatory cytokines (16). *NOD2 3020insC*, the mutation most common in CD, is an insertion that results in a premature stop codon. The truncated protein lacks the last leucine-rich repeat, which might affect ligand binding (11). Although macroautophagy up-regulation in response to ligand binding by other PAMP receptors has been documented (17), such a connection has not yet been reported for NOD2. Nevertheless, these genetic links suggest the possibility that altered macroautophagy promotes CD.

In this review, we will discuss potential mechanisms whereby macroautophagy can protect from CD by virtue of its influence on innate and adaptive immune responses.

CD and the innate immune response

CD is a chronic IBD, for which cause and cure remain elusive. At present, the available data mainly support a model, in which an aberrant immune response to commensal bacteria in the gut causes the disease. Anti-inflammatory drugs and immunosuppression relieve the symptoms, but patients frequently relapse and need surgery to remove the inflamed parts of the gut (18).

In the healthy gut, several innate immune mechanisms protect the gut from commensal and pathogenic bacteria (19). The epithelium forms a barrier that is further protected by a layer of mucus containing anti-microbial peptides (20). Paneth cells that are found near the base of the crypts of the small intestine play a major role in the secretion of anti-microbial peptides (Fig. 1). Additionally, macrophages and other innate as well as adaptive immune cells help keep the intestinal flora in check. In the inflamed gut of genetically predisposed CD patients, an aberrant immune response to commensal bacteria seems to cause chronic inflammation. Macroautophagy supports these mucosal innate immune mechanisms in various ways, as detailed below.

Macroautophagy in Paneth cells

First, we will discuss how CD-associated mutations and macroautophagy influence the biology of the Paneth cell. In mice with hypomorphic expression of *atg16L1* or from patients homozygous for *atg16L1T300A*, Paneth cells show abnormal secretion of their granules (21). Furthermore, Paneth cells from both species express elevated amounts of adipocytokines. However, it is unclear how Atg16L1 independently or as part of the classical macroautophagy pathway interacts with the exocytosis pathway and how it regulates transcription of genes that respond to gut injury.

The role of Paneth cells in gut inflammation is underscored by the finding that Paneth cells from patients carrying the CD-associated mutations in *NOD2* secrete reduced amounts of α-defensins (22). By compromising the function of Paneth
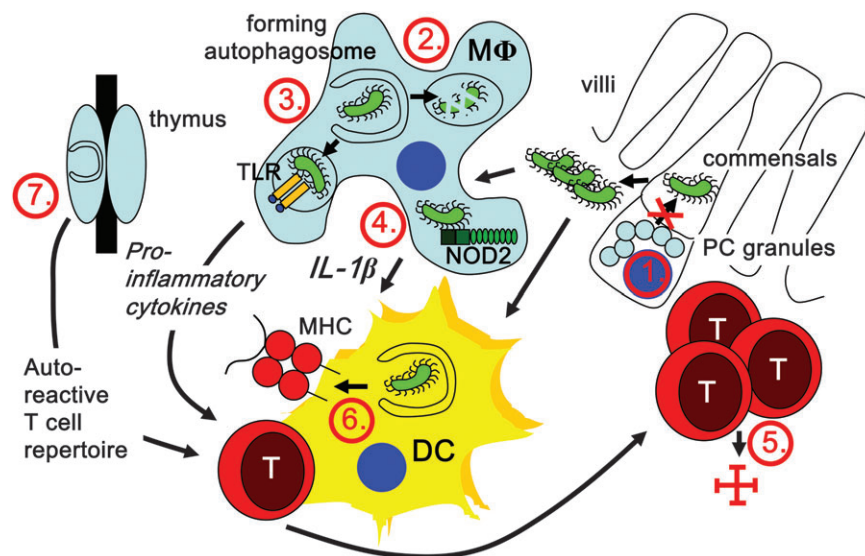

Fig. 1. Possible mechanisms by which mutations in macroautophagy genes could interfere with gut homeostasis and lead to IBD. Atg16L1 hypomorphic Paneth cells (PCs) secrete anti-microbial peptides less efficiently; (1) Atg16L1T300A-carrying PCs therefore might prevent commensal invasion less efficiently. (2) Macrophages (MΦ) with compromised macroautophagy might be attenuated in eliminating invading commensals; (3) they might fail to deliver commensal-derived PAMPs to TLRs for immune activation; (4) they might produce increased amounts of pro-inflammatory cytokines, like IL-1β, in response to cytosolic PAMP recognition. In addition to these innate immune recognition mechanisms, mutations in macroautophagy genes might also compromise adaptive immune responses. (5) T cell survival in the gut mucosa and therefore efficient restriction of invading commensals might be affected. (6) Macroautophagy-dependent endogenous MHC presentation of commensal-derived antigens by dendritic cells (DCs) could be compromised. (7) Finally, altered thymic T cell selection in patients with mutations in macroautophagy genes might favor a T cell repertoire with gut autoreactivity.

cells, mutations in the macroautophagy and NOD2 pathway affect the gut milieu, possibly allowing uninhibited expansion of resident microorganisms that could drive the inflammation seen in CD patients.

Macroautophagy in macrophages

The balance of the gut flora and the immune system may further be upset by the inability of innate immune cells to clear microorganisms. In macrophages, macroautophagy is critically involved in efficient degradation of intracellular bacteria. Both pathogens that condition phagosomes for their replication and those that escape into the cytosol can be restricted by macroautophagy. In murine macrophages infected with the parasite *Toxoplasma gondii*, Atg8 is recruited to the parasitophorous vacuole after CD40 stimulation, which induces fusion with lysosomes (23). In *T. gondii* as well as in *Mycobacterium tuberculosis* infection, macroautophagy can overcome bacterial evasion mechanisms and efficiently eliminate the pathogen (24, 25). Similarly, group A streptococci that escape the endosome for replication in the cytosol can be restricted by macroautophagy (26).

These results suggest that CD-associated mutations in macroautophagy genes could affect the ability of macrophages to clear intracellular bacteria. Indeed, human cell lines modified to only express Atg16L1T300A show impaired macroautophagy of *Salmonella typhimurium* (13). Diminished expression levels of *IRGM* also reduce macroautophagy of *S. typhimurium* in HeLa cells (9).

In addition to eliminating intracellular bacteria, components of the classical macroautophagy pathway might be involved in processing of extracellular pathogens after phagocytosis. If a particle activates Toll-like receptor (TLR) signaling while being phagocytosed, this recruits Atg8 to the phagosome in a murine macrophage cell line (27). Coating with Atg8 enhances fusion of the phagosomes with lysosomes after TLR2 activation and might have an impact on bacterial clearance. In this scenario, most organisms of the intestinal flora could be targeted by macroautophagy. Consequently, a defect in macrophage clearance of phagocytosed bacteria might contribute to an elevated bacterial load in the gut that could upset the gut equilibrium and result in inflammation.

Macroautophagy and PAMP recognition

Independent of phagocytosis, there is crosstalk between PAMP recognition and the macroautophagy pathway. On the one hand, TLR signaling induces macroautophagy. For example, incubation of primary human macrophages or murine macrophage cell lines with the TLR4 ligand LPS induces macroautophagy in a myeloid differentiation factor 88 (MyD88)-independent, Toll–IL-1 receptor domain-containing adaptor-inducing IFN-β-dependent pathway (28). Ligand binding to TLR1, TLR3, TLR5, TLR6 or TLR7 also induces macroautophagy as measured by the increase of green fluorescent protein–Atg8 punctae in the same cell line (29), which was confirmed for TLR3 and TLR7 by another study (17). In contrast to Xu *et al.* (28), Shi and Kehrl (29) found macroautophagy induction to be MyD88-dependent and provided mechanistic data on how the pathways intersect: TLR signaling frees Beclin-1 from the inhibitory complex with Bcl-2 consequently inducing macroautophagy. In addition, TLR signaling positively influences macroautophagy by inducing transcription of Atg genes (27).

On the other hand, macroautophagy can deliver intracellular TLR ligands to their respective endosomal TLRs. At least in plasmacytoid dendritic cells, efficient IFN-α production after vesicular stomatitis virus infection was dependent on macroautophagy, which delivers cytosolic single-stranded RNA to TLR7 (30). Similarly, TLR9 ligand targeting to endosomes by B cell receptors seems to be macroautophagy dependent (31).

In contrast to TLRs, much less is known about the influence of cytosolic PAMP receptor engagement on macroautophagy. The NOD-like protein IPAF (IL-1β-converting enzyme protease-activating factor), however, seems to inhibit macroautophagy during *Shigella* infection (32). Vice versa, components of the macroautophagy machinery block signaling of RNA helicases, for example the retinoic acid-inducible gene-I molecules, and macroautophagy knockout cells produce more type I IFN in response to infection with single-stranded RNA viruses, which engage this cytosolic PAMP recognition pathway (33, 34). Mutations in macroautophagy genes could therefore diminish TLR signaling or enhance cytosolic PAMP recognition. These effects could either inhibit efficient commensal control or increase inflammatory cytokine secretion, respectively. Both mechanisms might contribute to gut inflammation during CD.

Inflammatory cytokines in CD

Indeed, CD patients have elevated levels of inflammatory cytokines like IL-1, IL-6 and tumor necrosis factor (TNF) in the intestine and the serum (35, 36). IL-17 is also up-regulated in CD patients (35). However, involvement of IL-17 in the pathogenesis in CD has recently been challenged by data showing a protective function of IL-17A in intestinal inflammation (37). Furthermore, macrophages from CD patients were shown to spontaneously produce IL-12, which could explain the bias toward T_h1 cells in CD (38) and thereby the elevated levels of IFN-γ (39). Expression of the T_h2 cytokine IL-4 on the other hand is drastically reduced (38). Of note, transcription of IL-10, a cytokine known to down-regulate the expression of T_h1 cytokines and inhibit inflammation, is suppressed in patients carrying the CD-associated *NOD2* mutation (40).

As a possible explanation for this pathogenic cytokine milieu, murine LPS-stimulated macrophages deficient for Atg16L1 produce elevated levels of the pro-inflammatory cytokine IL-1β and readily promote gut inflammation after dextran sulfate sodium treatment (41). However, these mice do not spontaneously develop IBD or recapitulate macroautophagic activity of patients carrying the CD-associated *atg16L1T300A* mutation. In Atg16L1-deficient mice, macroautophagy is completely abrogated resulting in death soon after birth. The *atg16L1T300A* mutation, however, does not affect basal macroautophagy levels and causes more subtle effects than complete loss of function (13). It would therefore be interesting to study cytokine expression from macrophages in Atg16L1 hypomorphic or *atg16L1T300A* knock-in mice (21) in order to link CD-associated mutations

with specific defects in macroautophagy and cytokine production. Nevertheless, macroautophagy might negatively regulate pro-inflammatory cytokine secretion in the gut and CD-associated mutations could compromise this regulation.

CD and the T cell compartment

Clearly, adaptive immune responses also contribute to CD. In particular, T cells play a role in CD pathogenesis, which is accompanied by increased numbers of activated CD4$^+$ T cells in the gut of CD patients (36) and remission of CD in HIV-infected patients (42). In addition, IBD can be induced by adoptive transfer of purified CD4$^+$ T cell populations (43). Disease-inducing CD4$^+$ T cells are predominantly T$_h$1 cells. T cell responses seem to be specific for a small number of antigens; however, the antigens vary from patient to patient (36). In the following part, we will discuss how mutations in the macroautophagy pathway could affect homeostasis, function and repertoire selection of T cells and thereby predispose for CD.

T cell homeostasis

Murine and human T cells can up-regulate macroautophagy (44) and mainly need it for survival after development to single-positive thymocytes as well as after activation in peripheral tissues. T cells from *atg5*$^{-/-}$ chimeric mice develop normally, but T cell numbers are reduced in the thymus as well as in the periphery. This is due to increased apoptosis in peripheral CD8$^+$ T cells and diminished proliferation of CD4$^+$ and CD8$^+$ T cells after TCR stimulation (44). In contrast to a pro-survival role of macroautophagy described by Pua *et al.* (44), Li *et al.* (45) found macroautophagy to be involved in increased cell death after growth factor withdrawal in murine T$_h$2 cells. Furthermore, an autophagic death program can eliminate CD4$^+$ T cells after IFN-γ stimulation in the absence of *Irgm1*, the murine homologue of human *IRGM* (46).

With respect to CD4$^+$ T cell homeostasis, CD patients again do not show a phenotype as pronounced as the animal models discussed above. Nevertheless, these data suggest that mutations in CD-associated genes could affect survival of T cells, and it is tempting to speculate that subsets of T cells could be affected differently, for example with a survival effect on inflammation-promoting T$_h$17 cells and a pro-apoptotic effect on regulatory T cells, including T$_h$2-polarized IL-10-secreting cells.

Autophagy in antigen presentation

The role for autophagy in antigen presentation on MHC class II presentation is less speculative. We and others have shown that autophagy delivers antigens mainly from the cytoplasm into the MHC II-loading compartment in antigen-presenting cells (47–51). This results in enhanced antigen presentation of autophagy substrates on the cell surface, an increase in T cell activation and improved clearance of pathogens. Therefore, mutations in the macroautophagy pathway could affect how efficiently the adaptive immune system eliminates infections. Indeed, the *atg16L1T300A* mutation compromises MHC class II presentation of macroautophagy-targeted antigens (S. Meixlsperger and C. Münz, unpublished

data). Of note, mutations in macroautophagy genes could also inhibit extracellular antigen processing for MHC class II loading after phagocytosis (27). This could render priming of regulatory T cells and immune surveillance of commensal invasion more difficult.

In addition to the role of macroautophagy in MHC class II presentation, a recent report suggests that macroautophagy is also involved in the presentation of viral antigens on MHC class I molecules (52). English *et al.* noticed that, in late phases of infection, herpes simplex virus 1 (HSV-1)-infected murine macrophages were less capable of stimulating CD8$^+$ T cell responses after treatment with the macroautophagy inhibitor 3-methyladenine or knock-down of Atg5. Mild heat shock or administration of the pyrogenic cytokine IL-1β up-regulated this pathway, which resembled classical macroautophagy with respect to the formation of Atg8-coated, virus-containing vesicles that fuse with lysosomes. However, other aspects of this pathway that supports MHC class I presentation differed significantly from classical macroautophagy. Namely, the autophagosomal membranes originated from the nuclear envelope and formed four-layered membrane structures as well as the classical double-membrane-surrounded autophagosomes.

Future experiments will have to show if this macroautophagy-dependent pathway contributes to the control of infection *in vivo* and how its substrates gain access to the MHC class I antigen-processing machinery. Additionally, it will be important to analyze whether this pathway is specific for HSV-1 infection or also participates in the presentation of other antigens. Possibly, this additional, unconventional macroautophagy pathway might have evolved in adaptation to mechanisms used by HSV-1 to evade the classical macroautophagy pathway (53, 54). However, these data suggest that macroautophagy can also enhance MHC class I-restricted antigen presentation under certain conditions. Mutations in the macroautophagic machinery might therefore prohibit efficient MHC presentation for immune control of commensal invasion and for tissue-protective tolerance induction, failure of which could significantly contribute to CD.

Macroautophagy and T cell selection

One major mechanism of tolerance induction is based on the elimination of autoreactive T cells in the thymus. Recently, macroautophagy has been implicated in this central tolerance. Murine thymic epithelial cells (TECs), which select thymocytes that recognize self-MHC molecules and are self-tolerant, were described to show high levels of constitutive macroautophagy (55). This observation and the evidence for antigen presentation on MHC class II molecules after macroautophagy raised the possibility that macroautophagy delivers antigens to MHC class II molecules in TECs for positive and negative selection, shaping the T cell repertoire to self-MHC recognition and self-tolerance, respectively.

To test this hypothesis, Nedjic *et al.* (48) transplanted *atg5*$^{-/-}$ thymi into TCR transgenic or athymic recipient mice. *atg5*$^{-/-}$ thymi supported positive selection of some but not all CD4$^+$ T cell specificities and for all CD8$^+$ T cell specificities. In athymic nude mice, Atg5 deficiency shaped the T cell repertoire toward autoreactivity, resulting among other autoimmune signs in continuous gut inflammation. However, as most CD

patients develop symptoms only in their 20s, a general defect in T cell selection is unlikely. Also, the T cell repertoire seems to be normal in CD patients. Only within the lesions is the repertoire more restricted. Therefore, only distinct T cell specificities promoting bowel inflammation might not be deleted in thymi with mutated macroautophagy genes and promote disease only after recurrent re-stimulation.

Possible treatment of CD by manipulating macroautophagy

Macroautophagy is a central pathway for the function of innate and adaptive immune responses. As discussed, mutations of gene products in the pathway might affect multiple cell types and aspects of macroautophagy in CD. Therefore, manipulation of macroautophagy could have therapeutic merit for patients affected by this disease.

Rapamycin, a drug that is widely used to stimulate macroautophagy in experimental settings and that has been approved as an immunosuppressant after organ transplantation in humans, was recently used to treat a patient with CD after failure of standard medication (18). On a dose of 4 mg a day and in combination with an anti-TNF antibody, her symptoms improved markedly, abolishing the need for surgery. While this is very promising, the study needs to be extended to more patients and additional *in vitro* analysis needs to show whether the effect is due to up-regulation of macroautophagy or to a general immunosuppressive effect of rapamycin. Experiments in mice support the possibility of an effect of rapamycin on the up-regulation of macroautophagy *in vivo*. When Jagannath and colleagues immunized mice with rapamycin-treated, bacille Calmette–Guérin-infected dendritic cells, the mice controlled infection with virulent *M. tuberculosis* better than control mice did (47). Furthermore, macroautophagy stimulation with rapamycin prevented toxicity of neurodegenerative protein aggregates *in vivo* (56).

However, more selective methods than rapamycin need to be developed to up-regulate macroautophagy in distinct cell lineages that are involved in altered innate and adaptive immune responses in CD. In any case, it will be important to monitor side effects closely, as macroautophagy regulates survival and apoptosis of various cell types as well as turnover of proteins and organelles.

Conclusion

We are just beginning to understand what causes and maintains inflammation in CD. In addition to environmental factors, there is a big genetic contribution to the pathogenesis of CD and probably only a combination of mutations results in disease. Some mutations are common to IBDs, while others are exclusively associated with CD, like polymorphisms of *atg16L1*, *IRGM* and *NOD2*. Especially, the first two affect the macroautophagy pathway. In addition, it is tempting to speculate that *NOD2* mutations might compromise macroautophagy regulation upon bacterial constituent recognition, as has been shown for other PAMP receptors. Macroautophagy de-regulation affects the function of innate immune cells like Paneth cells and macrophages. It could also affect the T cell repertoire, antigen presentation and T cell homeostasis.

Most conclusions with respect to how mutations in macroautophagy genes could affect CD are based on the analysis of gene-deficient mice. However, these might only poorly recapitulate the situation in CD patients. For example, *IRGM* is a single gene in humans, but there are several *Irgm* family members in the mouse. Even though murine Irgm1 corresponds to human IRGM, other Irgm family members might compensate for Irgm1 deficiency in the mouse. More importantly, the CD-associated mutations do not abrogate protein expression. They affect expression levels or impair certain functions of the affected proteins. It will therefore be important to generate and study knock-in mice for CD-associated mutations and to confirm findings from murine models on patient samples. Furthermore, it is of interest to analyze immune cells of the gut microenvironment as opposed to immune cells from the periphery or cells differentiated from bone marrow precursors.

Understanding the molecular pathways inducing macroautophagy as well as knowing the downstream effectors will greatly enhance the possibility of manipulating the affected cells specifically. Atgs might also have functions distinct from the classical macroautophagy pathway (57) as has been suggested for Atg8 in phagocytosis (27). Since the macroautophagy pathway is associated with various other diseases, research into the role of macroautophagy in the pathogenesis of CD will not only benefit patients affected by this disease but also could affect treatment of neurodegenerative disorders, muscle diseases and cancer.

Abbreviations

atg5	autophagy-related gene 5
atg16L1	Atg16-like 1 gene
CD	Crohn's disease
GABA	γ-aminobutyric acid
GABARAP	GABA (A) receptor-associated protein
HSV-1	herpes simplex virus 1
IBD	inflammatory bowel disease
IFN-γ	IFN gamma
IRGM	immunity-related GTPase family M
MAP1LC	microtubule-associated protein 1 light chain
MyD88	myeloid differentiation factor 88
NOD2	nucleotide-binding oligomerization domain containing 2
PAMP	pathogen-associated molecular pattern
TEC	thymic epithelial cells
TLR	Toll-like receptor
TNF	tumor necrosis factor

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