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Lysozyme promotes the release of toll-like receptor-2 stimulants from Gram-positive but not Gram-negative intestinal bacteria

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We recently reported that intestinal concentrations of soluble stimulants of Toll-like receptor (TLR)-2 and TLR4 are markedly elevated in two murine models of inflammatory bowel disease (IBD); dextran-sodium-sulphate (DSS)-induced colitis and *Toxoplasma gondii*-induced ileitis. It was also shown that while enterobacterial species, such as *Escherichia coli*, released abundant soluble pro-inflammatory stimulants of macrophage TNFα secretion and TLR2-signalling into their growth environment, the release of such stimulants by Gram-positive organisms, including *Bifidobacterium bifidum*, *Lactobacillus plantarum* and *Enterococcus fecalis*, was approximately three orders of magnitude lower, leading to the proposal that Gram-positive commensals are unlikely to be major contributors of soluble pro-inflammatory TLR-stimulants during IBDs. In this addendum to the previous study, additional data are presented to address the question of whether elevated soluble TLR2-stimulants may derive from the action of intestinal lysozyme on the host micribiota via increased release of bacterial lipopeptides. It is shown that while lysozyme treatment of Gram-positive organisms, including *Lactobacillus plantarum* and *Enterococcus fecalis*, promotes the release of TLR2-stimulants from these organisms, lysozyme did not promote the release of soluble pro-inflammatory stimulants from two model intestinal Gram-negative organisms (*Escherichia coli* and *Bacteroides fragilis*), or from human fecal samples. The increase in TLR2-stimulants in murine colitis and ileitis therefore most likely reflects the overgrowth of enterobacterial species, rather than the action of lysozyme on Gram-positive bacteria.

Sequestration of TLR2-stimulants by the Gram-Positive Cell Wall is Reversed by Lysozyme

It is now widely accepted that inappropriate inflammatory responses directed against the host commensal microbiota play a key role in the initiation and propagation of IBDs.[1] As the TLRs represent a major family of innate immune receptors involved in the induction of inflammation, it has been proposed that, depending on the nature of the TLR-ligand and the cell-types involved, the stimulation of TLR-signalling in the intestine may contribute to the development of IBD.[2-4] Notably, the severity of colitis and markers of intestinal inflammation were shown to be reduced in mice deficient in TLR2 or TLR4 compared to wild-type animals in at least one model of DSS-colitis, suggesting that luminal stimulants of TLR2 or TLR4 could contribute to IBD pathology.[5] The observation that intestinal concentrations of soluble TLR2- and TLR4-stimulants are up to ~3,000-fold higher in murine IBDs therefore prompted two further questions. These were: (1) what is responsible for the increase in TLR-stimulants in the diseased gut and (2) could increased luminal concentrations of TLR-stimulants promote IBD pathology?

Our previous results suggested that the increase in luminal TLR4-stimulants is due to the overgrowth of enterobacterial species, which is a feature of both colitis and ileitis in mice,[5,6] since the biological

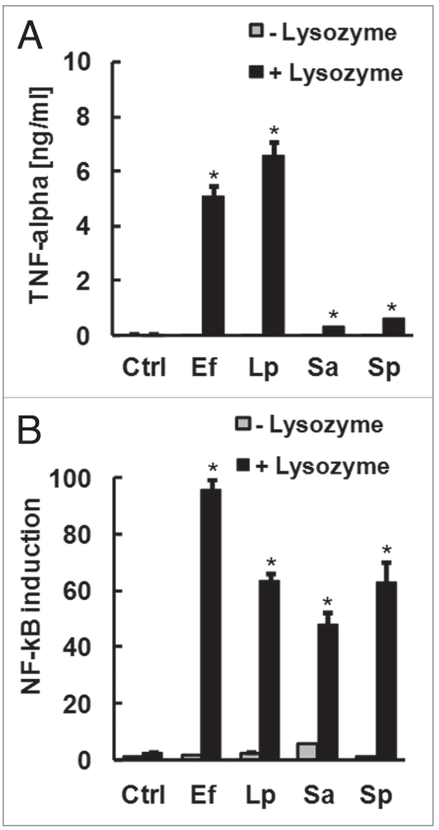

Figure 1. Lysozyme promotes the release of soluble TLR2 stimulants from Gram-positive bacteria. Gram-positive organisms (Ef, *E. fecalis*; Lp, *L. plantarum*; Sa, *S. aureus*; Sp, *S. pyogenes*) were heat-killed, resuspended in PBS at 10^9 bacteria/ml and treated with or without 1 mg/ml lysozyme for 2 h. Conditioned medium was then filter-sterilized, diluted 1:100 in tissue culture medium and applied to RAW macrophages for the measurement of secreted TNFα (A) or HEK-293 cells transfected with TLR2 and NFκB reporter for the measurement of TLR2-dependent NFκB signalling (B). *$p < 0.05$ vs. cells cultured in medium alone (Ctrl), ANOVA with Dunnet's test.

activity of intestinal TLR4-stimulants was blocked by the LPS-sequestering molecule polymyxin-B, and the LPS of various Bacteroides species did not stimulate TLR4-dependent signalling.[7] Enterobacterial overgrowth was also considered to be the likely cause of the increase in TLR2-stimulants, since enterobacterial species released approximately 1,000-fold more TLR2-stimulants into their growth environment than did a selection of Gram-positive intestinal organisms or Bacteroides spp.[7]

However, since several studies have shown that luminal concentrations of the peptidoglycan-degrading enzyme lysozyme can be up to 50-fold higher in subjects with Crohn disease or ulcerative colitis compared to healthy subjects,[8-10] the question of whether elevated lysozyme could contribute to the release of TLR2 stimulants from otherwise non-inflammatory Gram-positive commensals remained to be answered. To address this, a panel of Gram-positive organisms were heat-killed, resuspended in PBS and treated with or without hen-egg lysozyme for 2 h. The filtered supernatant of each culture was then analyzed for capacity to stimulate TLR2-signalling or cytokine production in macrophages. Lysozyme treatment of Gram-positive bacteria significantly increased the capacity of conditioned medium to stimulate macrophage TNFα secretion (**Fig. 1A**), and TLR2-dependent signalling in transfected HEK-293 cells (**Fig. 1B**). Taken together, these results suggest that the thick peptidoglycan layer of the Gram-positive cell wall may play a key role in the observed sequestration of pro-inflammatory lipopeptides from detection via the growth medium.

Effect of Lysozyme on Release of TLR-Stimulants by Gram-Negative Bacteria and Human Fecal Samples

We next aimed to establish if lysozyme also promoted the release of soluble pro-inflammatory stimulants from the Gram-negative organisms *E. coli* and *B. fragilis*. Surprisingly, lysozyme tended to reduce the capacity of supernatant of these organisms to stimulate macrophage TNFα production (**Fig. 2A**). This was accompanied by a reduction in the biological activity of TLR2-stimulants in the supernatant of these organisms (**Fig. 2B**). Pre-treatment with lysozyme also significantly reduced the capacity of LPS or the synthetic bacterial lipopeptide Pam_3CSK_4 to stimulate TLR2- or TLR4-signalling (**Fig. 2B and C**). This was not due to non-specific inhibition of TLR-signalling or NFκB activation, as concurrent treatment of cells with lysozyme and Pam_3CSK_4 or LPS without pre-incubation did not result in reduced TNFα production or NFκB activation (data not shown). Overnight incubation with lysozyme also reduced further the capacity of extracts to stimulate TLR-signalling compared to treatment for 2 h.

As lysozyme appeared to exert both pro- and anti-inflammatory effects on Gram-positive and Gram-negative bacterial suspensions, respectively, we next aimed to determine if lysozyme may modulate the capacity of the normal human fecal microbiota to release soluble pro-inflammatory TLR-stimulants. Lysozyme treatment of 5 human fecal samples for 2 h had little impact on soluble TLR2 and TLR4 stimulants in 3 of the samples, while two of the samples showed a significant reduction in soluble TLR2- or TLR4-stimulant activity, which was accompanied by a reduced capacity to promote macrophage TNFα production (**Fig. 3**).

Is Lysozyme Relevant to IBD Pathology?

The present findings suggest that lysozyme may play both pro- and anti-inflammatory roles in the context of the host microbiota and their products. Specifically, although lysozyme enhances the potential of Gram-positive bacteria to release TLR2-stimulants, other functions of lysozyme appear to reduce the biological activity of soluble lipopeptides and LPS once they are released. Although to our knowledge the capacity of lysozyme to reduce the activity of bacterial lipopeptides has not been reported previously, these findings are consistent with earlier reports that lysozyme binds to and reduces the biological activity of LPS in vitro and in vivo.[11,12] Further studies will be required to establish how lysozyme reduces the biological activity of lipopeptides.

These preliminary experiments also suggest that lysozyme does not necessarily increase the pro-inflammatory potential of the normal human fecal microbiota, but rather can in some instances reduce the capacity of such extracts to stimulate TLR-signalling or macrophage cytokine production. This suggests that under certain circumstances, intestinal lysozyme could exert an overall anti-inflammatory effect, as supported by the observation that oral supplementation with lysozyme reduces the inflammation and tissue damage associated with DSS-induced colitis in pigs.[13]

Are Soluble TLR-stimulant Concentrations Mediators or Markers of Murine IBD?

As the TLRs play key roles in the detection of bacterial products and the induction of inflammatory signalling, the roles played by TLRs in IBD have become the subject of intensive research. To date, the results of these studies have been largely conflicting, and have therefore become the subject of much debate. In essence, the point of discussion is that although in some models of murine IBD, TLR-signalling appears to play a key role in the maintenance of gut barrier integrity and protection against the development of IBDs,[14-16] in other models, TLR-signalling, particularly via TLR4, was shown to exacerbate existing IBD by promoting inflammation.[2-6,17]

Only recently have new data emerged to provide a tenable explanation for the conflicting results observed in earlier studies of TLR function in IBD. These results suggest that while TLR-signalling in intestinal epithelial cells promotes the enhancement of barrier function and therefore protects against disease,[14-16,18-20] TLR-signalling in cells of haematopoietic origin, particularly macrophages, may promote disease activity by responding to TLR-ligands with a pro-inflammatory response.[2,4,6,21,22] These differences in the ways that certain cell-types respond to PAMPs are evident in the demonstration that TLR-signalling in intestinal epithelial cells promotes the upregulation of barrier-enhancing tight junction proteins and antimicrobial peptides, rather than overt inflammatory responses.[14-16,18-20] By contrast, the stimulation of TLR-signalling in macrophages and other PAMP-sensitive cell types that reside beneath the intestinal epithelial layer can lead to the expression of pro-inflammatory cytokines, chemokines and other mediators that ultimately exacerbate IBD.[2,4,6,21,22]

These opposing and cell-type specific contributions of TLR-signalling to gut health are perhaps best exemplified by two very recent studies. Gong et al. showed that epithelial cell-specific inhibition of TLR-signalling via blockade of MyD88 results in chronic inflammation of the small intestine due to increased penetration of the microflora into the mucosa, where myeloid cells may then be exposed to bacterial products resulting in inflammation.[23] Taking the opposite approach, Asquith et al. showed that MyD88-dependent activation of myeloid cells, but not epithelial cells, was required for the development of chronic intestinal inflammation.[24] The emerging evidence therefore supports the notion that while TLR-signalling in epithelial cells is protective against IBD, TLR-signalling in myeloid cells can promote disease progression.

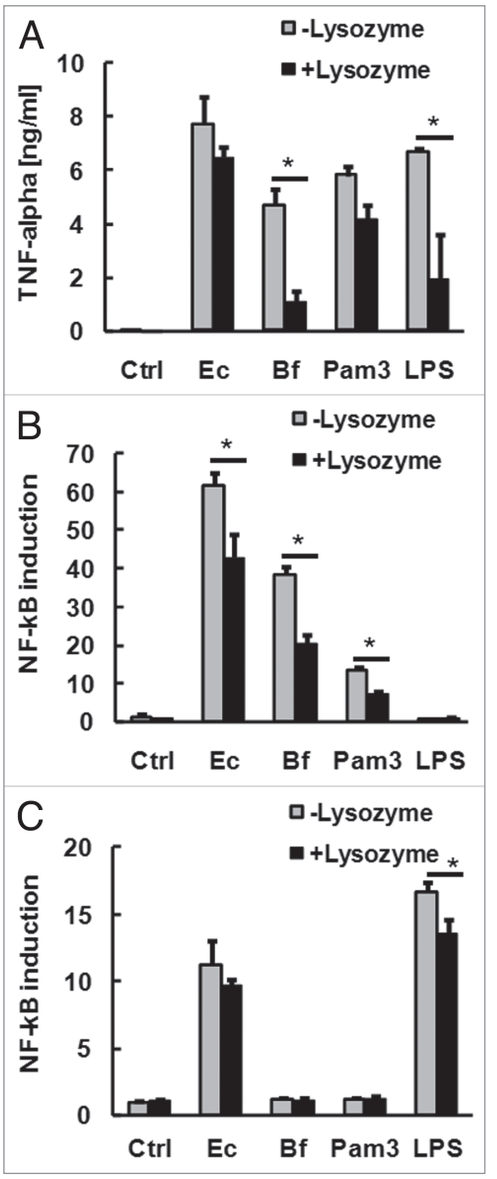

Figure 2. Effect of lysozyme on release of soluble TLR-stimulants from Gram-negative bacteria. *E. coli* (10^9/ml), *B. fragilis* (10^9/ml), Pam_3CSK_4 (1 μg/ml) and LPS (1 μg/ml) were resuspended in PBS with or without 1 mg/ml lysozyme for 2 h at 37°C. Filtered supernatants were then diluted 1:100 in tissue culture medium and applied to RAW macrophages for the measurement of secreted TNFα (A) or HEK-293 cells transfected with TLR2 (B) or TLR4/MD2 (C) for the measurement of TLR-dependent NFκB signalling. *$p < 0.05$ (ANOVA with Tukey's test).

These recent findings therefore add complexity to the question of whether or not elevated luminal concentrations of TLR-stimulants could contribute to the development of IBDs. Current evidence suggests that elevated concentrations of PAMPs in the intestine are not in themselves sufficient to initiate inflammatory disease, as the healthy, intact colon is largely unresponsive to exogenously applied flagellin, LPS or lipoteichoic acid,[21,22,25,26] and

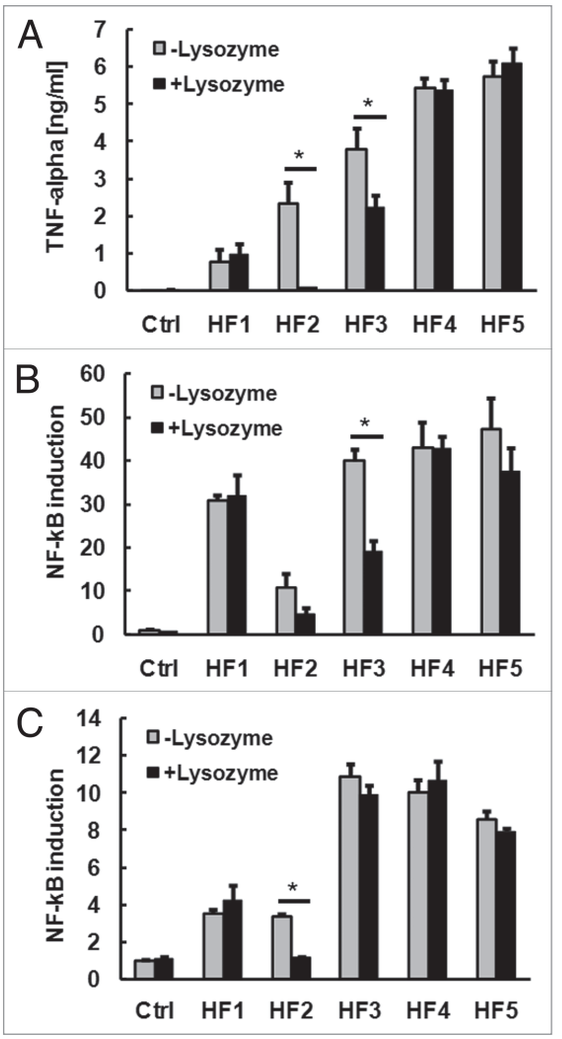

Figure 3. Effect of lysozyme on release of soluble TLR-stimulants from human fecal samples. Human fecal (HF) samples of 5 healthy subjects were resuspended in PBS (1:50 wt/vol) and treated with or without 1 mg/ml lysozyme for 2 h at 37°C. Filtered supernatants were then diluted 1:10 in tissue culture medium and applied to RAW macrophages for the measurement of TNFα production (A) or HEK-293 cells transfected with TLR2 (B) or TLR4 (C) for the measurement of TLR-dependent NFκB signalling. *p < 0.05 (ANOVA with Tukey's test).

we showed that mice with high PAMP concentrations in the ileum did not develop inflammation in the colon.[7] However, when the epithelial barrier is disrupted by agents such as DSS or infection, it has been shown that experimental administration of diverse PAMPs to the gut lumen can trigger inflammation[2,4,6,21,22] and increase severity of disease.[2-4] Taken together, these findings suggest that elevated concentrations of luminal TLR-stimulants could contribute to the inflammatory processes of IBDs if existing disease has caused damage to the epithelial layer.

The next question that requires to be addressed is whether or not intestinal concentrations of TLR2, TLR4 and TLR5 stimulants may be altered in human IBDs, such as Crohn disease and ulcerative colitis. If it turns out that intestinal TLR-stimulants are elevated in human disease, further studies may be warranted to investigate the therapeutic potential of inhibiting TLR-signalling for the remission of IBD. However, the emerging duality of TLR-function in the gut suggests that such approaches would have to balance carefully the requirement for epithelial stimulation by luminal PAMPs to maintain barrier integrity with the desired result of dampening mucosal inflammation.

Acknowledgements

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