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Dear Sir or Madam:

Enclosed is a report prepared for the UBC co-op office summarizing the functional effects of deficient Apolipoprotein E (ApoE) in the context of a *Salmonella* Typhimurium-induced colitis mouse model. The focus of our preliminary findings include the protective role lipoproteins play in impeding bacterial invasion of the gastrointestinal tract and the impaired recruitment of T cells due to the lack of functional ApoE. As the findings described in the report are still pre-mature, recommendations have been made to facilitate further investigation into the immunological consequences of lacking functional ApoE.

Sincerely,

Student

Summary

Apolipoprotein E (ApoE) deficient mice exhibit hyperlipoproteinemia, a condition typified by enhanced levels of lipoprotein in the serum. Lipoproteins have been shown to be protective against bacterial infection in mouse models through two mechanisms: interference with bacterial interactions with endothelial cells thus impeding organ invasion and neutralization of bacterial lipopolysaccharide that might otherwise induce potentially harmful inflammatory cytokines. Despite the protective qualities of lipoproteins, ApoE plays a key role in mediating natural killer T-cell (NKT) activation. The NKT cell system is crucial during the latter stages of infection because it bridges the progression from an innate immune response to acquired immunity. To examine the functional loss of ApoE, we studied *Salmonella* Typhimurium-induced colitis in ApoE deficient mice.

Our preliminary data indicates that at day 4 post-infection in our streptomycin pretreated *S*. Typhimurium-induced colitis model, the ApoE KO mice were less susceptible to bacterial invasion and colonization in the cecum and colon as compared to the WT control. Furthermore, the induction of cytokines (IFN-γ and TNF-α) indicative of an over exuberant and potentially harmful inflammatory response observed in the colons of the ApoE KO mice appeared diminished in comparison to the WT control. This data could provide evidence that the lipoproteins play a protective role in blocking bacterial invasion and nullifying LPS.

In the context of a prolonged infection as demonstrated in our survival curve, the ApoE KO mice appeared to have impaired recovery displaying increased mortality and morbidity as compared to the WT control. This could be a result of decreased ApoE mediated recruitment of NKT cells that is crucial for bacterial clearance.

Repetition of experiments are needed to confirm reproducibility while additional approaches are needed to evaluate the systemic nature of the infection and histophathological changes to the gastrointestinal organs. Flow cytometry can also be done to evaluate the immune cell population mobilized in the ApoE deficient mice in comparison to the WT control to provide insight in the mechanisms of the observed impairment in recovery.

Introduction

Salmonella enterica is a gram-negative facultative intracellular anaerobe responsible for 1.3 billion cases of diseases globally each year (Coburn *et al.* 2006). *Salmonella* subspecies can be divided into serovars characterized by flagellar, carbohydrate, and lipopolysaccharide structures (Coburn *et al.* 2006). *S. enterica* is able to infect a number of hosts while disease manifestation is dependent on the susceptibility of the host and the infectious serovar (Coburn *et al.* 2006). This pathogen is capable of causing one of four conditions including typhoid fever, enterocolitis or diarrhea, bacteremia, and chronic asymptomatic carriage (Coburn *et al.* 2006). For instance, *S. enterica* Serovar Typhimurium (*S.* Typhimurium) causes enterocolitis in humans and cattle, but a systemic infection in mice (Grassl and Finlay 2008). *S.* Typhimurium infections in humans are typically acquired by ingestion of contaminated food or water (Grassl and Finlay 2008). In the early stages of infection, the pathogen can colonize the intestine and mediate the subsequent uptake and transcytosis of the bacteria across the epithelium into the lamina propria through the induction of cytoskeletal rearrangements of intestinal epithelial cells (Grassl and Finlay 2008 and Kum *et al.* 2009).

Apolipoportein E (ApoE) plays an important role in the degradation of lipoprotein constituents (Roselaar and Daugherty 1998). Thus, defects in ApoE can result in hyperlipoproteinemia, a condition characterized by increased levels of lipoproteins in the plasma (Netea *et al.* 2009). Lipoproteins are known to be protective against *Salmonella* infection in mice models by interfering with host cell binding (Netea *et al.* 2009). By preventing *Salmonella* interactions with endothelial cells and monocytes, organ invasion is impeded (Netea *et al.* 2009). These lipoproteins may also neutralize bacterial lipopolysaccharide (LPS), preventing the induction of potentially harmful pro-inflammatory cytokines such as TNF-α that are necessary for the internalization of *Salmonella* by host cells (de Bont *et al.* 2000 and Netea *et al.* 2009).

In spite of the suggested protective role of increased lipoproteins, mice deficient in ApoE have displayed an impaired response to bacterial infection (Roselaar *et al.* 1998 and de Bont *et al.* 2000). It has been suggested that ApoE is involved in the activation of natural killer T-cells (NKT) by acting as a molecular chaperone in the delivery of

Abbreviations used in report: IFN, interferon; TFN, tumor necrosis factor; MCP, monocyte chemoattractant protein; LDLR, low density lipoprotein receptor; APC, antigen presenting cell

bacterial antigens to APCs (Chida *et al*. 2004). NKT cell activation is followed by secretion of IFN-g and IL-4, which are Th1 and Th2 cytokines, respectively (Chida *et al*. 2004). Because of their crucial functional link between the innate and the adaptive immune systems, the NKT cell population is an important target for infectious diseases (Taniguchi *et al*. 2003).

The research on the cellular and molecular mechanisms of *Salmonella*-induced intestinal inflammation has been accelerated by the utilization of an acute gastroenteritis model in mice, which involves the oral infection of mice with *S.* Typhimurium following pre-treatment with streptomycin (Kum *et al.* 2009). Following the infection, the mice demonstrate signs of intestinal inflammation, which are most evident in the cecum and share many of the pathological features of *Salmonella* enterocolitis in humans (Kum *et al.* 2009). In this study, we explore the functional properties of ApoE deficiency involved in host response to *S.* Typhimurium infection in a colitis-induced mouse model.

Materials and Methods

Bacterial Culture

Salmonella enterica serovar Typhimurium SL1344 was grown with overnight shaking (200 rpm) in Luria-Bertani broth supplemented with 50 μg/ml streptomycin at 37˚C for 18 h.

Mouse Expermients

Wild-type (WT) C57Bl/6 (9-10 weeks) and ApoE KO (9-10 weeks) were pretreated with 20 mg streptomycin 24 h prior to infection with 3 x 10^6 *Salmonella* Typhiumurium SL1344 by oral gavage. Mice were sacrificed on Day 1 and Day 4 post-infection. Colon and cecum were harvested for bacterial enumeration while the colon extracts were used for cytokine analysis.

Bacterial Enumeration

Spleens and colons were harvested at Day 1 and Day 4 post-infection, weighed, and collected in 1 ml of sterile PBS and homogenized with an MM 301 mixer mill (Retsch). Serial dilutions of the homogenates were plated on Luria-Bertani agar plates with 100 μg/ml streptomycin. Plates were incubated for 24 h at 37˚C.

Colonic cytokine measurement

Colon samples were harvested and homogenized as described in the bacterial enumeration. Homogenates were spun twice at 13 200 rpm for 30 min each at 4˚C to remove insoluble matter. Colon supernatants were assayed for cytokines using the mouse inflammation cytometric bead array (CBA) assay kit (BD Biosciences).

Statistical Analysis

Bacterial enumeration and CBA assays for cytokines of infected mice were compared using two-tailed, unpaired *t* test. Analyses were performed with a 95% confidence interval using GraphPad Prism software.

Discussion

Increased levels of lipoprotein may offer protection against Salmonella colonization in cecum and colon at 4 days post-infection

The preliminary bacterial enumerations of the colon harvested on Day 1 and Day 4 post-infection suggest that a loss of ApoE function resulting in a state of hyperlipoproteinemia may offer protection against invasion in the *Salmonella* infection model. There were significantly lower colony forming units (CFU) in the colon of ApoE KO mice as compared to the WT on Day 1 and Day 4 post-infection. Moreover, significantly lower CFU was found in the cecum of ApoE KO mice in comparison with the WT on Day 4 post-infection.

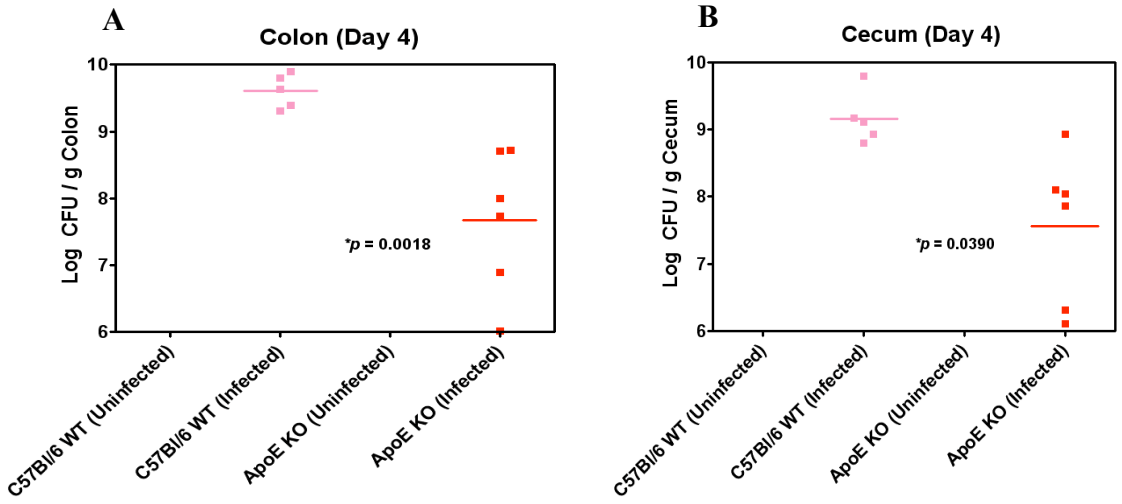

FIGURE 1. *S.* Typhimurium colonization in the colon and cecum is diminished in the ApoE deficient mice at Day 1 and Day 4 post-infection. Mice lacking ApoE and WT controls were treated with streptomycin prior to oral infection with *S.* Typhimurium and sacrificed 4 days post-infection. Bacterial loads harvested from the cecum ($p>0.005$) and the colon ($p>0.05$) had significantly decreased as compared with those of infected WT controls.

S. Typhimurium induced ApoE KO mice has reduced secretion levels of TNF-α, IFN-γ, and MCP-1 as compared with WT infected mice at 4 days post-infection

Our CBA assay indicates that *S*. Typhimurium infected ApoE KO mice had significantly reduced levels of secreted proinflammatory cytokines (A, B, C) in comparison with the WT control.

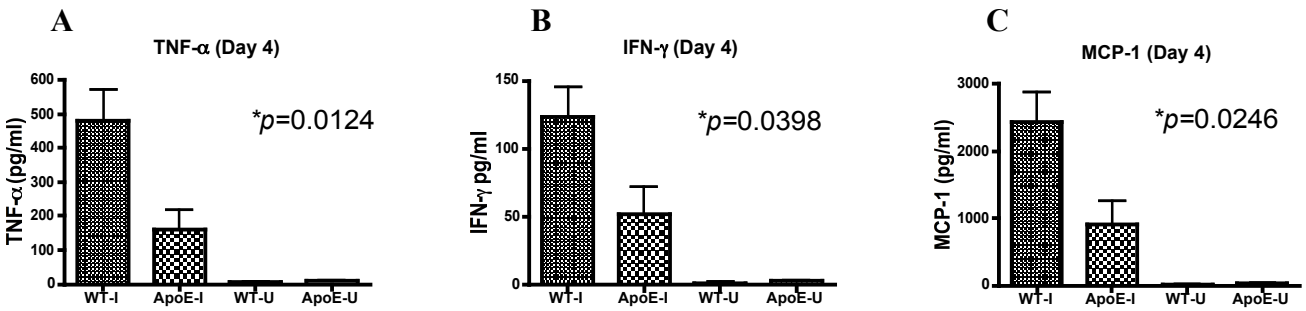

FIGURE 2. Cytokine concentrations in the colon extracts were determined by CBA assay. Significantly reduced concentrations of TNF-α ($p>0.05$) (A), IFN-γ ($p>0.05$) (B), and MCP-1 ($p>0.05$) (C) were observed in the infected ApoE Day 4 post-infection as compared to the WT infected.

To further investigate the role of lipoproteins in neutralizing LPS and the subsequent induction of cytokines, we compared the proinflammatory cytokine profiles of ApoE KO and WT infected mice. Typically in a *Salmonella* infection, the LPS plays a role in both TLR2 and TLR4 induced productions of cytokines, including TNF-α (Tapping *et al* 2000). Moreover, IFN-γ is a proinflammatory cytokines that is produced in response to LPS induced activation of lymphocytes commonly observed in Gram-negative bacterial infection models (Mattner *et al.* 2005). However, MCP-1 is produced by immune and non-immune cells in response to the presence of other cytokines including TNF (Zisman *et al.* 1997). The reduced secretion of these cytokines as indicated by our assays may suggest that the harmful inflammation that is typically observed, as in our WT controls, is significantly reduced thus facilitating the initial clearance of the infection.

S. Typhimurium infected ApoE deficient mice display increased mortality as compared to WT infected mice

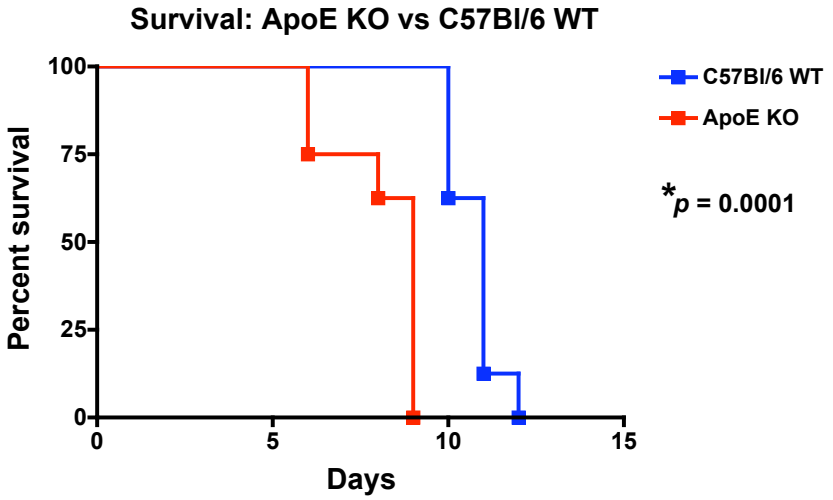

FIGURE 3. WT control mice and ApoE KO mice were treated with streptomycin before infection with *S.* Typhimurium and monitored for signs of morbidity and mortality. Significantly higher mortality rates were observed in the ApoE KO mice ($p < 0.0001$).

In contradiction with the protective role of lipoprotein in the invasion and colonization of *Salmonella* in the cecum and the colon suggested by the bacterial enumeration data and the cytokine profile, the survival curve indicates that the *Salmonella* infected ApoE deficient mice display increased morbidity and mortality as compared to the WT control. However, this impaired ability to recover from infection is evident beginning at day 6 post-infection, a time point that is beyond the relevance of study in the context of an acute infection in the colitis model used in the study. This impaired recovery may be the result of decreased activation of NKT cells due to the lack of ApoE, thus breaking the progression of the innate to the acquired or delayed immune response (Taniguchi *et al.* 2003).

Conclusion

(1) The preliminary data suggests that the increase in lipoproteins found in the ApoE KO mice may be functionally important in impeding the invasiveness of *Salmonella* during infection. This may be achieved by two means: the increased presence of lipoproteins that interfere with bacteria binding to host cells thus preventing organ invasion and the neutralization of bacterial lipopolysaccharides which prevents the subsequent induction of harmful proinflammatory cytokines.

(2) The survival curve indicates an impaired recovery from infection in the ApoE deficient mice as compared to the WT control despite initially having reduced levels of bacterial invasion in the cecum and the colon. This could be due to the lack of ApoE mediated NKT cell recruitment.

Recommendations

Though this study is still in its preliminary stages, data obtained thus far indicates contradicting findings pertaining to the host susceptibility of ApoE deficient mice in a *S.* Typhimurium induced colitis system. The most curious aspect of our results is the significantly impaired ability of the ApoE deficient mice to recover despite being more resistant to bacterial invasion and colonization of the colon and the cecum. To investigate the suggested role of ApoE in mediating NKT cell activation we could determine and compare the immune cell type population of the infected ApoE KO mice with the WT control using flow cytometry. Following the acute phase of infection in our colitis model, we could expect to see a reduced number of NKT cells in the ApoE deficient mice.

In addition to the repetition of these experiments to confirm repeatability, additional approaches will be used to compare the loss of ApoE in a *Salmonella* infection model with infected WT controls including the following: (1) bacterial enumeration of *S.* Typhimurium in the spleen and serum to evaluate the systemic nature of the infection (2) evaluation of histopathological changes in the cecum (3) neutrophil detection in the cecum using myeloperoxidase (MPO) immunofluorescent staining.

While the role of ApoE in recruiting NKT cell during *Salmonella*-induced colitis is an interesting topic worth further investigation, we can also specifically study the effects of increased levels of lipoproteins in the serum by using LDLR KO mice, which also exhibit hyperlipoproteinemia, in the same *S.* Typhimurium induced colitis model (Netea *et al.* 2009).

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