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ELIMINATION OF C5aR PREVENTS INTESTINAL MUCOSAL DAMAGE AND ATTENUATES NEUTROPHIL INFILTRATION IN LOCAL AND REMOTE ORGANS

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Abstract

The complement C5a pathway has been shown to be an important mediator of inflammation and tissue injury. To further understand the role of C5a receptor (C5aR) pathway in ischemia/reperfusion (I/R) injury, and to evaluate the potential of antagonizing C5aR to protect from I/R injury, we tested the effect of eliminating C5aR using C5aR knockout (KO) mice and their wild-type (WT) littermates in a superior mesenteric artery occlusion (SMAO) intestinal I/R injury model. C5aR KO and WT mice were subjected to SMAO or sham for 45 min. After 3 h of reperfusion, the percentage of injured ileal villi was twice as high in WT mice subjected to SMAO as compared with the C5aR KO mice. In addition, the number of neutrophils was 34% higher in WT mice subjected to SMAO as compared with the C5aR KO mice. Moreover, ileum and lung myeloperoxidase activities after SMAO were significantly higher in WT than C5aR KO mice. Apoptotic cell death was induced after reperfusion in WT-SMAO and was reduced by more than 50% in C5aR KO mice. The plasma level of TNF- α was increased ~3.74-fold in WT subjected to SMAO compared with sham. In contrast, the level was increased only ~1.18-fold in the C5aR KO mice subjected to SMAO. In conclusion, this study demonstrates that elimination of the C5aR pathway protects the intestine from I/R injury and diminishes intestine-derived pulmonary neutrophil sequestration. Blocking C5aR may be considered as a potential therapeutic intervention for I/R injury.

Keywords

Complement pathway; knockout mice; ischemia/reperfusion injury; superior mesenteric artery occlusion; gut I/R injury

INTRODUCTION

The need to restore normal blood flow to prevent tissue injury from oxygen deprivation is a common theme in acute clinical settings that involve ischemia. While restoration of blood flow in this setting is critical, reperfusion may further augment tissue damage (1). Reperfusion injury is characterized by the release of free radicals, microvascular

dysfunction, and infiltration of immune cells and inflammatory mediators, resulting in further damage to both local and/or remote tissues (2,3).

The complement system is a well-accepted mediator of inflammation and ischemia/reperfusion (I/R) injury and exerts its effects in a number of ways (4,5). For example, complement activation leads to the release of proinflammatory anaphylatoxins (C3a and C5a) and the tissue-injurious terminal membrane attack complement complex C5b-9. In the context of complement activation, C5a seems to be especially important, because C5a can induce neutrophil attraction and aggregation, chemotaxis, cytotoxic activity, and the release of reactive oxygen metabolites and proteases. In addition, C5a is a potent chemotactic factor for eosinophils, basophils, monocytes/macrophages, and microglial cells and is responsible for the upregulation of vascular adhesion molecules. The biological activity of C5a is mediated by the interaction with its receptor C5aR that is widely expressed in both inflammatory and noninflammatory cells (6).

The role of C5 downstream pathway in preclinical models of intestinal, hepatic, and renal I/R injury has been evaluated using C5-deficient mice and antagonists of C5 and C5aR (7–11). Inhibition of C5 activation by an anti-C5 antibody administered to wild-type (WT) mice subjected to mesenteric I/R prevented C5a generation, polymorphonuclear cell infiltration, and deposition of the terminal complement complex on damaged tissues in a manner similar to that observed in C5-deficient mice (12–16). These studies, however, did not distinguish between the actions of C5a and the C5b-9 terminal complex. Using a pharmacological approach, Fleming et al. (9) attempted to distinguish the actions of C5a and C5b-9 terminal complex by using a C5aR antagonist in WT mice subjected to mesenteric I/R injury. In their study, when C5aR antagonist was administered 5 min before reperfusion, the intestinal damage decreased significantly compared with WT mice subjected to I/R injury without the antagonist; however, the C5aR antagonist did not completely prevent I/R-induced injury. Furthermore, neutrophil infiltration in the intestine and remote organs was not examined.

Consequently, to further study the role of neutrophil infiltration in intestinal I/R injury and its regulation by C5aR, we used a genetic approach using C5aR knockout (KO) mice. In previous studies, these mice have been used to demonstrate the requirement of C5aR for host defense and the synergistic role it plays with its ligand (C5a) and other inflammatory mediators such as TNF- α and IL-6 in immune complex-mediated peritonitis and skin injury (17,18).

The present study is the first to evaluate C5aR KO mice subjected to I/R injury. In this study, we adopted the superior mesenteric artery occlusion (SMAO) model, which is considered to be a golden “proof of concept” model for I/R injury (19) and conducted a comparison between WT and C5aR KO mice to evaluate the role of C5aR-mediated pathway, using key disease markers such as mucosal damage, neutrophil infiltration to local and remote organs, circulating cytokine levels, and apoptotic cell death.

MATERIALS AND METHODS

Animal preparation

The heterozygous C5aR KO mice [strain *C.129S4(B6)-C5aR^{tm1Cge}/J*] were licensed from the Children’s Hospital Boston and purchased from the Jackson Laboratory (Bar Harbor, Maine). The heterozygous mice were intercrossed to generate homozygous KO mice. The correct homozygous (C5aR^{-/-}) pups were identified by polymerase chain reaction (PCR) genotyping according to the protocol from the Jackson Laboratory. The C57BL/6 mice were used as WT control and were also purchased from the Jackson Laboratory. Male homozygous KO and WT mice aged between 12 and 14 weeks were used for the *in vivo*

study. Animal study protocols were approved by the New Jersey Medical School Animal Care and Use Committee and the Novo Nordisk Ethic Review Committee. The experiments were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals.

Intestinal I/R injury model

The model for SMAO was used as previously described (20) and is briefly summarized as follows. Mice were anesthetized intraperitoneally with pentobarbital (70 mg/kg). Through a midline laparotomy, the superior mesenteric artery (SMA) was isolated and temporarily occluded by placing a 4-0 suture around the SMA at its origin from the aorta. An immediate blanching of the small intestine and the cecum confirmed that the blood supply to these intestinal segments was completely shut off. The abdomen was then covered with a sterile moist gauze pad. After 45 min of intestinal ischemia, the ligature was removed around the SMA. After verifying return of blood supply to the intestine, the laparotomy incision was closed. There are four groups of animals: (i) C5aR KO-SMAO: C5aR KO mice subjected to SMAO procedure; (ii) WT-SMAO: WT mice subjected to SMAO procedure; (iii) C5aR KO-sham; and (iv) WT-sham. The mice subjected to sham SMAO were anesthetized and were performed a 3-cm laparotomy, and their SMA was looped with a 4-0 ligature; however, the artery was not occluded. Three hours after reperfusion, sham or SMAO mice were sacrificed, and the tissue was harvested. Whole blood was collected through a cardiac puncture in the syringe containing 0.1 mL of 100 U/mL heparinized saline at sacrifice, and then the blood was centrifuged at 1,500 revolutions per min for 15 min, and the resulted plasma was stored at -80°C .

Histopathology

At sacrifice, a segment of the terminal ileum (1 cm) was excised and fixed in 10% buffered formalin. Semi-thin (4 μm) sections were then cut and stained with hematoxylin-eosin. Five random fields with 100 to 250 villi from each animal were then scored for injury in a blinded fashion using light microscopy at 100 $\times$ magnification (21).

Intestinal edema

A 15-cm piece of intestine was taken from the mice and weighed for wet weight after brief cleaning, and then the intestine was placed at 60°C for 48 h and weighed again for dry weight. The ratio is expressed as wet weight divided by dry weight.

Myeloperoxidase activity in tissue homogenates

Three centimeters of ileum was taken by direct snap-freezing in liquid nitrogen for 5 min and then stored at -80°C . In addition, whole lung and a piece of liver tissue were taken and prepared in a similar way. Neutrophil sequestration was quantitated by myeloperoxidase (MPO) activity (22). Myeloperoxidase is considered a biochemical marker for neutrophils as neutrophils contain 5% MPO in total protein. Briefly, the tissue samples (40–50 mg) were homogenized for 30 s in 1 mL of 20 mM potassium phosphate buffer (pH 7.4) and centrifuged for 30 min at 40,000 g at 4°C . The pellet was resuspended in 1 mL of 50 mM potassium phosphate buffer (pH 6) containing 0.5 g/dL hexadecyltrimethyl ammonium bromide. Samples were sonicated for 90 s, incubated at 60°C for 2 h, and then centrifuged. A supernatant sample (100 μL) was added to 2.9 mL of 50 mM potassium phosphate buffer (pH 6), containing 0.167 mg/mL *O*-dianisidine and 0.0005% hydrogen peroxide. Absorbance at 460 nm (A_{460}) was measured for 3 min.

Apoptotic cell death

Tissue apoptotic cell death was examined using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, Calif). Formalin-fixed paraffin-embedded tissue sections were deparaffinized with xylene followed by absolute ethanol, 95% ethanol, and 70% ethanol. The sections were washed with phosphate-buffered saline (PBS; 5 min) before treatment with protease K (20 µg/mL) for 15 min. After two washes with dH₂O for 2 min each, sections were quenched in 3.0% hydrogen peroxidase in PBS for 5 min at room temperature and rinsed twice with PBS for 5 min each time. Equilibration buffer was immediately applied directly to the sections for 20 s at room temperature. Terminal deoxynucleotidyl transferase enzyme was pipetted onto the sections followed by incubation in a humidified chamber for 1 h at 37°C. Slides were placed in a Coplin jar containing working strength stop/wash buffer and incubated for 10 min at room temperature. After rinsing three times in PBS, the sections were incubated with antidigoxigenin conjugate in a humidified chamber for 30 min at room temperature. The sections were rinsed with PBS, and color was developed in peroxidase substrate (AEC Substrate Kit; BD Biosciences Pharmingen, San Diego, Calif). Sections were rinsed in dH₂O and counterstained with hematoxylin (Sigma, St Louis, Mo). The specimens were dehydrated and mounted under a glass coverslip. Samples were then examined with bright field microscopy.

Plasma levels of cytokines

Detection of plasma cytokine levels was conducted with the Bio-Plex System (Bio-Rad Laboratories, Hercules, Calif) following the manufacturer's instruction, and the data analysis was performed with the Bio-Plex Manager software. All assays were carried out in a 96-well sterile filter plate (Cytokine Reagent Kit, Bio-Rad Laboratories) at room temperature and protected from light. Mouse cytokine customized three-plex panel (TNF- α , IL-6, and IL-1 β) and all reagents were purchased from Bio-Rad Laboratories. Standard curves were always generated before sample determinations. The plasma samples were diluted 4-fold with serum sample diluent. Each sample was determined in duplication and expressed in mean.

C5aR expression by quantitative reverse transcription–polymerase chain reaction

One centimeter of ileum was taken by direct snap-freezing in liquid nitrogen for 5 min and then stored at –80°C. The tissue was then homogenized in lysis buffer (from the RNA Isolation Kit; Qiagen, Valencia, Calif) for 30 s in medium speed using tissue-ruptor homogenizer. The homogenized tissue extracts were frozen at –80°C. RNA was isolated from tissue samples using the Illustra RNAspin Mini Kit (GE, Piscataway, NJ). Two micrograms of the RNA obtained was reverse transcribed using Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, Calif). One hundred sixty nanograms of cDNA was used as template for reverse transcription–PCR, and the reaction was run for 40 cycles. GAPDH (part# 4352932E, GenBank NM_008085; Applied Biosystems, Foster City, Calif) was used as the internal control. The mC5aR Taqman Primer/Probe sets were purchased from Applied Biosystems (part# 00500292_s1, NM_007577). The experiments and analysis were performed using the ABI 7500SDS System (Applied Biosystems).

Statistical analysis

All data are expressed as mean $\pm$ SD. Statistical analysis was performed with one-way ANOVA using GraphPad InStat software. Significance was defined as $P < 0.05$.

RESULTS

Intestinal mucosal damage was prevented in the C5aR-deficient mice

Histological scoring of the mucosal structure was performed. Representative histological images of villi structure are shown in Figure 1A, and the percent injured villi for the four animal groups is plotted in Figure 1B. Mucosal damage in WT mice subjected to SMAO procedure was significantly greater compared with the moderate or mild mucosal damage observed in most villi structures of the C5aR KO mice, suggesting that elimination of C5aR remarkably prevented villi injury in this SMAO model.

Intestinal edema was reduced in C5aR-deficient mice

To evaluate the intestinal edema, the ratio of wet to dry weights of isolated intestinal tissue was determined. Significant intestinal edema was evident in the WT-SMAO group, whereas the edema was markedly attenuated in the C5aR KO-SMAO group (Fig. 1C). The results suggest that the elimination of C5aR reduces intestinal edema.

Intestinal neutrophil infiltration was attenuated in the C5aR-deficient mice

Neutrophil infiltration in intestinal tissue and remote organs was examined 3 h after reperfusion by two methods: by histological examination and by determination of MPO activity. The number of neutrophils per 100 villi identified by histological examination was significantly higher in the WT-SMAO group than in the C5aR KO-SMAO group (Fig. 2), and this was accompanied with a significant increase in ileal MPO activity in WT-SMAO as compared with the C5aR KO-SMAO groups (Fig. 3A). These results indicate that a significant number of neutrophils had infiltrated the intestine after 3 h of reperfusion and that elimination of C5aR greatly reduced the number of neutrophils infiltrating into the intestine.

Next, we examined whether neutrophil infiltration into remote organs had increased after 3 h of blood reperfusion by measuring lung and liver tissue MPO activity. Lung MPO activity was significantly elevated in the WT mice as compared with the C5aR KO mice subjected to SMAO (Fig. 3B). Liver MPO activity was also significantly increased in the WT-SMAO group; however, MPO activity in the liver was not significantly attenuated in C5aR KO-SMAO mice compared with WT-SMAO mice (Fig. 3C).

Apoptotic cell death in intestine was attenuated in the C5aR-deficient mice

We evaluated next if apoptotic cell death contributed to villi damage by TUNEL assay. Among the four animal groups, only the WT-SMAO group displayed significant numbers of apoptotic cells along the villi, and the other three groups displayed minimal numbers of apoptotic cells. Representative stainings of the villi structures are shown in Figure 4A. Stained cells were found mostly in epithelial and goblet (gland) cells. Multiple cross and longitudinal sections were examined for each animal, and the mean numbers of apoptotic cells per cross and longitudinal section for the four animal groups are plotted in Figure 4B. Apoptotic cell death was induced after I/R injury in both WT-SMAO and C5aR KO-SMAO and was significantly reduced in C5aR KO mice compared with WT mice.

Intestinal I/R injury induced cytokine release into circulation was prevented in C5aR-deficient mice

To evaluate if cytokines are released into plasma and involved in systematic effects, the levels of proinflammatory cytokines including TNF- α and IL-6 were determined in the plasma samples. The mean levels of plasma TNF- α and IL-6 were significantly increased in the WT mice 3 h after the reperfusion, although there was variability in response among

individual animals (Fig. 5, A and B). In contrast, C5aR KO mice subjected to the same I/R procedure did not show an increase in plasma levels of TNF- α (Fig. 5A). The IL-6 level in C5aR KO mice subjected to SMAO also showed a trend of attenuation, although it was not statistically significant (Fig. 5B). Therefore, the plasma levels of TNF- α and IL-6 were elevated shortly after the intestinal I/R injury, and elimination of C5aR seemed to block this increase. The IL-1 β level was also determined, and we found no statistical difference among the four groups of animals (data not shown).

Expression of C5aR was moderately increased after I/R injury

Expression of C5aR has been reported to be upregulated in a number of acute disease settings (23–25). To address if there is an alteration of C5aR expression after the intestinal I/R injury, we conducted quantitative PCR and immunostaining in the WT animals subjected to sham or SMAO to determine the expression at both mRNA and protein levels. C5aR mRNA expression level was increased in WT-SMAO compared with WT-sham by ~75% 3 h after the reperfusion (Fig. 6). Immunostaining of the tissues collected at the same time point revealed a wide range of expression levels of C5aR protein in the WT-SMAO group. Some tissue samples from individual WT-SMAO animals showed significantly increased C5aR staining, whereas others revealed similar staining levels as compared with the WT-sham group (data not shown). Later time points need to be assessed for the expression level of C5aR protein.

DISCUSSION

Intestinal I/R after occlusion of the SMA has been used as a model to evaluate the involvement of pathways and mechanisms in I/R injury. Other studies have evaluated the role of the complement system using this model. For example, using an SMAO model, treatment with soluble CR1 attenuated the local and remote organ injury after intestinal I/R in rat (26). Furthermore, a C5aR antagonist administered before intestinal I/R injury has been shown to reduce mucosal damage. These pharmacological manipulations suggest that blocking multiple points in the complement pathway could prevent I/R injury.

In this study, we adopted a genetic model and demonstrated that C5aR deficiency reduced I/R injury. Our data suggest that elimination of C5aR (i) attenuates neutrophil infiltration in intestine, (ii) reduces MPO activity in local and remote organs, (iii) reduces intestinal edema, (iv) reduces apoptotic cell death, and (v) attenuates the elevated plasma level of proinflammatory cytokines after I/R injury.

Neutrophil infiltration has previously been suggested to mediate local tissue damage. Fleming et al. (9) have shown significant reduction in infiltrated polymorphonuclear neutrophils (PMNs) in an intestinal I/R injury model using C5^{-/-} mice. Treatment with 1 μ g of C5a did not increase neutrophil infiltration in C5^{-/-} mice, suggesting that activation of the receptor for C5a, but not the pharmacological concentration of C5a, may be the controlling point (9). Their study, combined with our data, clearly illustrates that signaling via the C5aR pathway leads to neutrophil infiltration and that the availability and activation of the receptor for C5a are crucial in recruiting neutrophils into local organs.

The early involvement of both local and systematic proinflammatory cytokines after intestinal I/R injury has been demonstrated by various groups (27–29). Grotz et al. (20) have reported that cytokine released from the gut correlates with the magnitude of injury caused by SMAO. Recently, proinflammatory cytokines TNF- α , IL-6, and IL-1 β were found to be elevated in rat plasma after intestinal I/R injury as early as 2 h after reperfusion (30). In our study using C5aR WT mice, TNF- α and IL-6 were increased in the plasma 3 h after reperfusion in the intestinal I/R injury model, which is in agreement with the results from

Rocourt et al. (30). We found that variation in plasma cytokine level is large among individual animals in the same treatment group. This might imply that there are differences in the magnitude of the responses among individual animals or that 3 h may be too early to observe a consistent systematic elevation of cytokines; if so, the variation in response among individuals probably would be significantly reduced at later time points. Nonetheless, we have demonstrated for the first time *in vivo* that elimination of C5aR attenuates systematic TNF- α elevation. This suggests that the C5a pathway can regulate levels of proinflammatory cytokines released from intestinal neutrophils. It is likely that local cytokine levels were attenuated as well in the intestine of C5aR-deficient mice; however, further experiments are needed to confirm this hypothesis.

The effect of C5aR elimination was evaluated in both local and remote organs. It is evident that neutrophils were recruited to lungs after I/R injury, and elimination of C5aR significantly attenuated this process based on the results of MPO activity in the lungs. However, at the same time point, C5aR deficiency did not significantly block neutrophil infiltration in liver. Moreover, we measured plasma alanine aminotransferase (ALT) activity as an index for liver damage. I/R injury caused a significant elevation of plasma ALT activity 3 h after reperfusion; however, we observed that elimination of C5aR had no significant effect on plasma ALT activity (data not shown). These data suggest that involvement of C5a pathway in different remote organs varies and that the downstream signaling pathways might not be identical. Samples collected at additional time points and examination of more mediators will add to our understanding of the temporal and spatial regulation of the C5aR signaling pathway.

Epithelial apoptotic cell death has been shown to be central to cell death in I/R injury (31–33). It is evident in our study that elimination of C5aR significantly reduced the number of apoptotic cells after I/R injury. Possible mechanisms by which the C5a pathway could regulate apoptosis include both indirect and direct mechanisms (4). In the indirect mechanism, activation of the C5a pathway could regulate apoptosis by stimulating proinflammatory cytokines such as TNF- α , and other mediators such as reactive oxygen in neutrophils. Release of these mediators into the local tissues, in turn, leads to apoptosis of the epithelial cells (5). The C5a pathway could also directly act on the intestinal epithelial cells and induce apoptosis by activating the JNK and p38 signaling pathways, resulting in the activation of caspase 3, as demonstrated by previous studies (34,35). Our studies showed a correlation between apoptotic cell death and neutrophil infiltration, thereby supporting the indirect mechanism of regulation. However, our data do not rule out the co-existence of the direct mechanism, and this awaits further investigation.

In summary, our studies support the hypothesis that the C5a pathway plays an important role in I/R injury. This pathway could have multiple effects including recruiting neutrophils to local and remote organs and inducing apoptosis in intestinal epithelial cells. Elimination of the C5aR is beneficial in reducing reperfusion-related injury and supports the potential use of C5aR antagonists as therapeutic approaches in acute clinical settings involving I/R.

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FIG. 1. A, Representative ileum sections from WT and C5aR KO mice after I/R injury Hematoxylin-eosin stainings of villi from (I) WT-sham, (II) WT-SMAO, (III) C5aR KO-sham, and (IV) C5aR KO-SMAO. Original magnification $\times 100$. **B, Percent villi injury after I/R injury.** Villi damage was scored by counting five random fields with 100 to 250 villi from each animal in a blinded fashion using light microscopy at $100\times$ magnification. Percent villi injury was determined by dividing the number of injured villi by the total number of villi examined multiplied by 100. *Significant difference between WT-SMAO and all other groups ($P < 0.01$). **Significant difference between WT-SMAO and C5aR-SMAO ($P < 0.01$). Number of animals examined: $n = 7$ in WT-sham, $n = 9$ in WT-SMAO, $n = 9$ in C5aR KO-sham, and $n = 10$ in C5aR KO-SMAO. **C, Intestinal edema after I/R**

injury. Intestinal edema was calculated by the ratio of wet to dry weight. Bars represent mean wet to dry ratio in each animal group $\pm$ SD. *Significant difference between WT-sham and WT-SMAO ($P < 0.001$). **Significant difference between WT-SMAO and KO-SMAO ($P < 0.01$). Number of animals examined: $n = 7$ in WT-sham, $n = 10$ in WT-SMAO, $n = 9$ in C5aR KO-sham, and $n = 12$ in C5aR KO-SMAO.

FIG. 2. Neutrophil infiltration in intestinal tissue after I/R injury

Neutrophil infiltration was analyzed histologically by counting the number of neutrophils from the crypt to the tip of villi in five random fields. More than 200 villi were examined for each animal, and the number of neutrophils in 100 villi was plotted for each group of animals. *Significant difference between WT-sham and WT-SMAO ($P < 0.001$).

**Significant difference between WT-SMAO and KO-SMAO ($P < 0.05$). Number of animals examined: $n = 7$ in WT-sham, $n = 9$ in WT-SMAO, $n = 9$ in C5aR KO-sham, and $n = 10$ in C5aR KO-SMAO.

FIG. 3. Myeloperoxidase activity in ileum (A), lung (B), and liver (C) after intestinal I/R injury
 Myeloperoxidase activity per milligram of tissue was calculated as follows: MPO activity (mU/mg tissue) = ($\delta A_{460} \times 13.5$)/weight (mg), where δA_{460} equals the rate of change in absorbance at 460 nm between 1 and 3 min. Bars represent mean MPO activity in each animal group $\pm$ SD. *Significant difference between WT-sham and WT-SMAO ($P < 0.001$). **Significant difference between WT-SMAO and KO-SMAO ($P < 0.05$). Animal number: $n = 7$ in WT-sham, $n = 10$ in WT-SMAO, $n = 9$ in C5aR KO-sham, and $n = 12$ in C5aR KO-SMAO. In liver, the difference between the WT-SMAO and KO-SMAO is not significant ($P = 0.07$).

FIG. 4. A, Representative ileum sections showing apoptotic cell death after I/R injury

The representative staining in villi structure from (I) WT-sham, (II) WT-SMAO, (III) C5aR KO-sham, and (IV) C5aR KO-SMAO is illustrated. Most stained cells were epithelial or gland cells. **B, The number of apoptotic cells was counted in high-power fields ($\times 400$).**

Stained cells in the villi but not the intestinal wall were included. Number of apoptotic cells per section in (I) cross and (II) longitudinal sections are plotted separately. Each bar represents the mean number of apoptotic cells per section $\pm$ SD. I. For cross sections, the number of sections examined per group of mice was as follows: WT-sham, 5 sections from three mice; WT-SMAO, 7 sections from four mice; KO-sham, 8 sections from five mice; and KO-SMAO, 14 sections from nine mice. II. For longitudinal sections, the number of sections examined per group of mice was as follows: WT-sham, 5 sections from three mice; WT-SMAO, 13 sections from seven mice; KO-sham: 13 sections from six mice; and KO-SMAO, 17 sections from nine mice. *Significant difference between WT-sham and WT-SMAO ($P < 0.05$). **Significant difference between WT-SMAO and KO-SMAO ($P < 0.05$).

FIG. 5. Plasma TNF- α (A) and IL-6 (B) levels after I/R injury

Plasma cytokine level from each animal was determined and graphed as scattered plot with the line indicating the mean level for each group. *Significant difference between WT-sham and WT-SMAO ($P < 0.05$). **Significant difference between WT-SMAO and C5aR KO-SMAO groups ($P < 0.05$).

FIG. 6. C5aR mRNA expression level in intestine after I/R injury

Reverse transcription-PCR product from intestinal tissue RNA was quantified, and C5aR mRNA expression was compared between the WT-sham and the WT-SMAO group. Each bar represents relative fold expression: all the absolute levels were justified against the mRNA expression levels of the internal control GAPDH in the same sample. The lowest mRNA expression level is defined as 1. The error bars indicate SD. There is a significant difference between WT-sham and WT-SMAO groups ($P < 0.05$).