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Decay-Accelerating Factor Attenuates C-Reactive Protein-Potentiated Tissue Injury After Mesenteric Ischemia/Reperfusion

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Background. **C-reactive protein (CRP) is an acute pro-inflammatory mediator that has been demonstrated to enhance ischemia/reperfusion (IR) injury by virtue of activating the complement system. CRP is able to interact with complement proteins such as C1q, complement factor H, and C4b-binding protein. Since complement activation is central in the expression of tissue injury following IR, we have investigated the effects of human decay-accelerating factor (DAF), a complement inhibitor, on CRP-potentiated complement activation and tissue injury in mice subjected to mesenteric IR.**

Materials and Methods. **Male C57B1/6 mice were allocated into eight groups: (1) Sham-operated group without IR injury; (2) CRP+Sham group; (3) IR group; (4) CRP+IR group; (5) DAF group; (6) CRP+DAF group; (7) IR+DAF group, and (8) CRP+IR+DAF group. Intestinal and lung injury, neutrophil infiltration, myeloperoxidase (MPO) expression, complement component deposition, and interleukin-6 (IL-6) production were assessed for each treatment group of mice.**

Results. **We report that administration of DAF significantly attenuates the CRP-enhanced intestinal injury as well as remote lung damages following acute mesenteric IR in mice, while DAF inhibits complement activation, suppresses neutrophil infiltration, and reduces IL-6 production.**

Conclusions. **Our study suggests that inhibition complement activation with DAF may prove useful for the treatment of post-ischemic inflammatory injuries associated with an increased production of CRP.** Published by Elsevier Inc.

Key Words: **complement; C-reactive protein; inflammation; rodent; mesenteric ischemia.**

INTRODUCTION

Ischemia/reperfusion (IR) represents a model of tissue injury in which circulation is reinstalled in an organ transiently deprived of blood flow. The ischemic insult alters the affected tissue making it susceptible to inflammatory damage during reperfusion. Furthermore, mediators produced in the ischemic areas diffuse when circulation is restored causing inflammation in remote organs not exposed to ischemia [1]. Several molecular and cellular mechanisms have been implicated in IR. These include mainly elements of the innate immune response, such as reactive oxygen species, cytokines, and chemokines, complement, natural antibodies, and neutrophils [1]. More recently, elements of the adaptive immune response have been shown to play significant roles. Specifically, T [2] and B cells [3] have been shown to be directly involved in the expression of tissue injury and depletion of either T or B cells prior to mesenteric IR limit both local and remote tissue injury. Complement (C) activation and neutrophil stimulation represent two major pathogenetic processes of IR-induced organ dysfunction, and local and remote tissue injury [4, 5]. Suppression of the C activities either by C inhibitors or in C-deficient animals has been demonstrated to attenuate tissue injury in various IR animal models. Studies which have demonstrated that C inhibition reduces IR tissue injury in various organs, including myocardium [6], lung [7], liver [8], intestine [9], kidney [10], and skeletal muscle

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[9], clearly identify the C system as a promising therapeutic target.

C-reactive protein (CRP), an acute phase reactant, which can increase in the blood plasma up to 1000-fold following inflammation, infection, or tissue injury in patients and animals, is part of the innate system as well as the C [11]. Unlike humans, CRP is not an acute-phase protein in mice [12, 13]. Accordingly, administration of human CRP to mice is a good model to study its functions. Mesenteric IR in rats induced endogenous CRP expression, and its deposition correlated with the deposition of C3, suggesting a role for CRP in the C activation [14]. We have reported that exogenous human CRP augments intestinal injury in a C-dependent manner in murine model of mesenteric IR [15]. These results suggest that CRP augmented IR-induced gut injury is strongly associated with C activation, and therefore implicate that C regulatory proteins or inhibitors would reduce tissue injury enhanced by CRP.

Decay-accelerating factor (DAF), a ubiquitously expressed intrinsic C-regulatory protein, is a glycosylphosphatidylinositol (GPI)-anchored membrane associated C regulatory protein, and is known to protect host tissue from autologous C activation. DAF contains four short consensus repeats (SCR) for binding CD97 and C3, C5 convertases and a heavily O-glycosylated region rich in serine and threonine. DAF inhibits C activation at the C3 and C5 convertase levels after binding to the convertases through its SCR2, SCR3, and SCR4 in both of classic and alternative C pathways, thereby limiting local C3a/C5a and subsequent blocking C5b-9 (MAC) production [16, 17].

We have shown that soluble human DAF attenuates local intestinal and remote lung injury in mice subjected to mesenteric IR [18]. Since human and rodent DAFs are not species restricted in their complement-inhibiting activity [19], we hypothesized that IR animals treated with human DAF would down-regulate human CRP-amplified C activity and effectively ameliorate tissue injury induced by mesenteric IR. We report in this study that CRP potentiates IR-triggered intestinal injury as well as remote lung damage, whereas the treatment with human DAF remarkably attenuates the CRP-induced injury *via* inhibition of complement activation and cytokine release. Our work supports the concept of utilizing C inhibitor as a therapeutic approach for IR related injury.

MATERIALS AND METHODS

Mouse Mesenteric IR Model

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adhered to principles stated in the Guide for the Care and Use Laboratory Animals. All procedures were reviewed and approved by the Institute's Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Male C57Bl/6 mice, aged 8 to 12 wk (Jackson Laboratory, Bar Harbor, ME) underwent at least 7-d acclimatization prior to experimentation.

Purified, sodium azide-free human CRP was obtained from US Biological (Swampscott, MA) and diluted to working concentrations in Tris-buffered saline (TBS, 10 mM Tris, 15 mM NaCl, 2 mM $CaCl_2$). To confirm purity, 1 and 5 μg of CRP were loaded on SDS-PAGE gel followed by using Silver and Coomassie Blue staining, which revealed a single 24 kD band. Endotoxin was not detectable (<0.03 EU/mL) in aliquots of CRP solution (1 mg/mL) as measured with Limulus Amebocyte Lysate Test Kit (Cambrex, East Rutherford, NJ).

Mice were assigned to the following experimental groups: (1) Sham (control, TBS injection, without mesenteric IR); (2) CRP+Sham; (3) IR; (4) CRP+IR; (5) DAF; (6) CRP+DAF; (7) IR+DAF, and (8) CRP+IR+DAF (n = 5–8 mice/group). Briefly, a midline laparotomy was performed followed by a 30-min equilibration period. The superior mesenteric artery was isolated and a small nontraumatic vascular clamp (Roboz Surgical Instruments, Gaithersburg, MD) was applied for 30 min. After this ischemic phase the clamp was removed, the laparotomy incisions were sutured and the intestine was reperfused for 2 h. Two hours prior to surgery, animals were injected i.p. with 0.25 mg/animal of purified human CRP or an equivalent volume of sterile TBS. Mice were anesthetized with ketamine (16 mg/kg) and xylazine (8 mg/kg) injected by i.p. All procedures were performed with the animal breathing spontaneously and maintained on 37 °C water-circulating heating pad. Animals were subjected to mesenteric I/R as previously described [20]. For DAF treatment, 5 min prior to reperfusion, mice were injected with either 2 μg per animal DAF (rhCD55/DAF; R and D Systems, Minneapolis, MN) or 0.2 mL sterile saline *via* tail vein injection. Mice were under anesthesia during the experiment and before euthanasia.

Serum and Tissue Collection and Preparation

Blood samples were collected by cardiac puncture at the time of euthanasia. Serum was separated immediately and stored at –80 °C for later analysis. After euthanasia the small intestine and lung specimens were removed, and each organ collected was immediately divided into three fractions, one was stored at –80 °C, the others were separately fixed in 10% buffered formalin phosphate and 4% paraformaldehyde.

The 4% paraformaldehyde-fixed tissues were washed twice (5 min each) in cold PBS, then sunk into 20% sucrose in PBS on a rocker in 4 °C-cold room for 2 h. The tissues were embedded in Tissue Tek Cryomold Standard Vinyl Specimen Disposable mold with Tissue Tek O.C.T compound (Sakura Finetek USA, Inc., Torrance, CA), and frozen on dry-ice. The frozen blocks were stored at –80 °C until section cut. Frozen blocks were cut at 5-μm sections using a cryostat and mounted on poly-L-lysine-coated slides.

Histopathology and Immunohistochemistry

The 10% formalin-fixed tissues were embedded in paraffin, sectioned at 5 μm transversely, and stained with hematoxylin and eosin (H & E). For each intestinal section, at least 50 villi were graded on a six-tiered scale for mucosal damage score and the mean score was recorded as described previously [21]. A score of 0 was assigned to a normal villus; villi with tip distortion were scored as 1; villi lacking goblet cells and containing Gugenheim's spaces were scored as 2; villi with patchy disruption of the epithelial cells were scored as 3; villi with exposed but intact lamina propria and epithelial cell sloughing were assigned a score of 4; villi in which the lamina propria was exuding were scored as 5; and villi displaying hemorrhage or denuded villi were scored as 6. All histologic analysis was performed in a blinded manner.

Severity of lung injury was scored in a method described by Nishina *et al.* [22]. Briefly, lung was scored by using a 5-point scale according to combined assessments of alveolar congestion, hemorrhage,

infiltration or aggregation of neutrophils in airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation: 0 = normal, 1 = minimum damage, 2 = mild damage, 3 = moderate damage, 4 = severe damage, and 5 = maximum damage. Pictures were taken on the Olympus Leica microscope at 400× magnification along the perimeter of each lung section.

Frozen slides were dried at room temperature, then fixed in cold acetone for 5–10 min. After another air-dry for 30 min, slides were washed with PBS and blocked for 30 min with 2.5% bovine serum albumin solution in PBS. Slides were then incubated with various primary antibodies (goat anti-mouse C3 IgG; MP Biomedical LLC, Solon, OH; rat anti-mouse C5a; BD Biosciences, San Jose, CA; rabbit anti-complement C5b-9 polyclonal Ab; Calbiochem, La Jolla, CA; goat anti-human DAF/CD55, R and D Systems, Minneapolis, MN; rat anti-mouse IL-6, Beckman Coulter, Fullerton, CA; rat anti-mouse neutrophils; AbD Serotec, Raleigh NC; rabbit anti-CD32; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100–1:1000 dilution for 60 min. Slides were incubated with appropriate secondary antibodies for 1 h (donkey anti-goat IgG-Alexa 488, Donkey anti-rabbit IgG-Alexa 594, goat-anti-rat IgG-Alexa 594 secondary antibodies, Invitrogen, Carlsbad, CA). Finally, slides were mounted with ProLong Gold antifade, counterstained with DAPI (4',6'-diamidino-2-phenylindole; Invitrogen, Carlsbad, CA), and observed under a confocal laser scanning microscope (Radiance 2100; Bio-Rad, Hercules, CA). Recorded digital images were processed using Image J software (NIH Bethesda, MD).

Western Blotting

Extracted proteins (20 ~ 50 μg) from frozen intestinal and lung tissue were separated in SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Pierce, Rockford IL). The membranes were blocked with 5% nonfat dry milk in TBST buffer for 1 h, and then incubated with primary antibodies (chicken anti-mouse C3/C3a; AbD Serotec, Raleigh NC; goat anti-mouse C5/C5a; Quidel, San Diego; CA; rabbit anti-rat C9; gift from Dr. B. Paul Morgan; goat anti-mouse MPO; R and D Systems, Minneapolis, MN) at 1:1000-1:2000 dilution for 1 h, followed by incubation with appropriate HRP-conjugated secondary Abs (goat anti-chicken IgY-HRP; AbD Serotec, Raleigh NC; donkey anti-goat IgG-HRP, goat anti-rabbit IgG-HRP; Santa Cruz Biotechnology, Santa Cruz, CA;) at 1:000–1:3000 dilution for 1 h. Specific bands were visualized by an ECL method (Amersham Biosciences, Piscataway, NJ) and captured with Fujifilm LAS-3000 System Configured for Chemiluminescence (Fujifilm Life Science, Edison, NJ). The density of each band was measured using QuantityOne Software (Bio-Rad).

ELISA

The serum concentrations of exogenous human CRP and endogenous mouse interleukin-6 (IL-6) were determined by an ultra-sensitive human-CRP specific enzyme-linked immunosorbent assay (ELISA) (Diagnostic Systems Laboratories, Webster TX) and a mouse IL-6 ELISA kit (eBioscience, San Diego, CA), respectively, according to the manufacturer's protocols.

Statistical Analysis

Data were expressed as mean ± SD unless otherwise stated. The two-tailed *t*-test or one-way ANOVA with Tukey's *post-hoc* test were performed using GraphPad Prism ver. 4 (GraphPad Software, San Diego, CA). P value < 0.05 was considered as significant.

RESULTS

DAF Prevents Enhanced Local Intestinal IR Injury Due to CRP

Our recent study showed that CRP administration (250–1000 μg/mouse) amplified the mesenteric IR-induced intestinal damage [15]. In this study, we pretreated mice with human CRP (250 μg/mouse, i.p.) at 2.5 h prior to ischemia. The concentrations of human CRP in murine sera were monitored at 30 min after of the occlusion of mesenteric artery, and 2 h after reperfusion using human CRP-specific ELISA kit. No significant differences were found in serum CRP levels in the CRP-sham (33.55 ± 5.24 μg/mL, $n = 7$) and CRP-IR (37.79 ± 8.13 μg/mL, $n = 8$) groups. Human CRP was not detected in animals without treatment of CRP.

Human DAF deposition in the intestinal and lung tissue was determined by specific immunostaining in DAF-treated mice due to its characteristic binding to membrane. We noticed a marked deposition of DAF in the gut and lung tissue in DAF-treated animals 2 h after injection, whereas DAF was not detected in the tissues of mice which did not receive DAF (unpublished results).

The mucosal damage induced by IR in various t groups was assessed histologically by H and E staining and microscopic observation using an established scale. As demonstrated in Fig. 1A and B, IR-induced intestinal injury was enhanced in animals that received human CRP. The injury score was 2.61 ± 0.42 in the CRP+IR group compared with 1.88 ± 0.08 in the IR group ($P < 0.001$). Treatment with DAF (2 μg/mouse, i.v., 5 min prior to reperfusion) not only reduced the damage induced by IR (the DAF+IR: 1.43 ± 0.13 *versus* the IR, $P < 0.05$), but also significantly attenuated the CRP-mediated enhancement of the IR-induced injury (the CRP+DAF+IR: 1.50 ± 0.09 *versus* the CRP+IR, $P < 0.001$). DAF did not completely prevent local injury in mice subjected to IR as gut damage scores in DAF-treated and DAF+CRP-treated IR mice were still markedly higher than those in relevant Sham groups (DAF + IR *versus* Sham: 1.43 ± 0.13 *versus* 0.40 ± 0.08, $P < 0.001$; DAF+CRP+IR *versus* CRP: 1.50 ± 0.09 *versus* 0.51 ± 0.10, $P < 0.001$).

DAF Ameliorates Lung Tissue Injury Following CRP-Augmented Intestinal IR

The mesenteric IR model was used for determining whether the protective effect of DAF treatment can be extended to remote or systemic injury after local IR. The lung is particularly susceptible to damage in the setting of intestinal ischemia [23]. Following intestinal IR, lung sections were analyzed to assess the severity of histological injury (Fig. 2A and B). In animals exposed to mesenteric IR, H and E staining revealed a great number of alveolar septal and interstitial capillaries that were congested with erythrocytes, and alveolar walls were thickened with neutrophil infiltration and edema compared with the Sham group. The average damage score in the animals subjected to IR was increased by 6.7-fold of

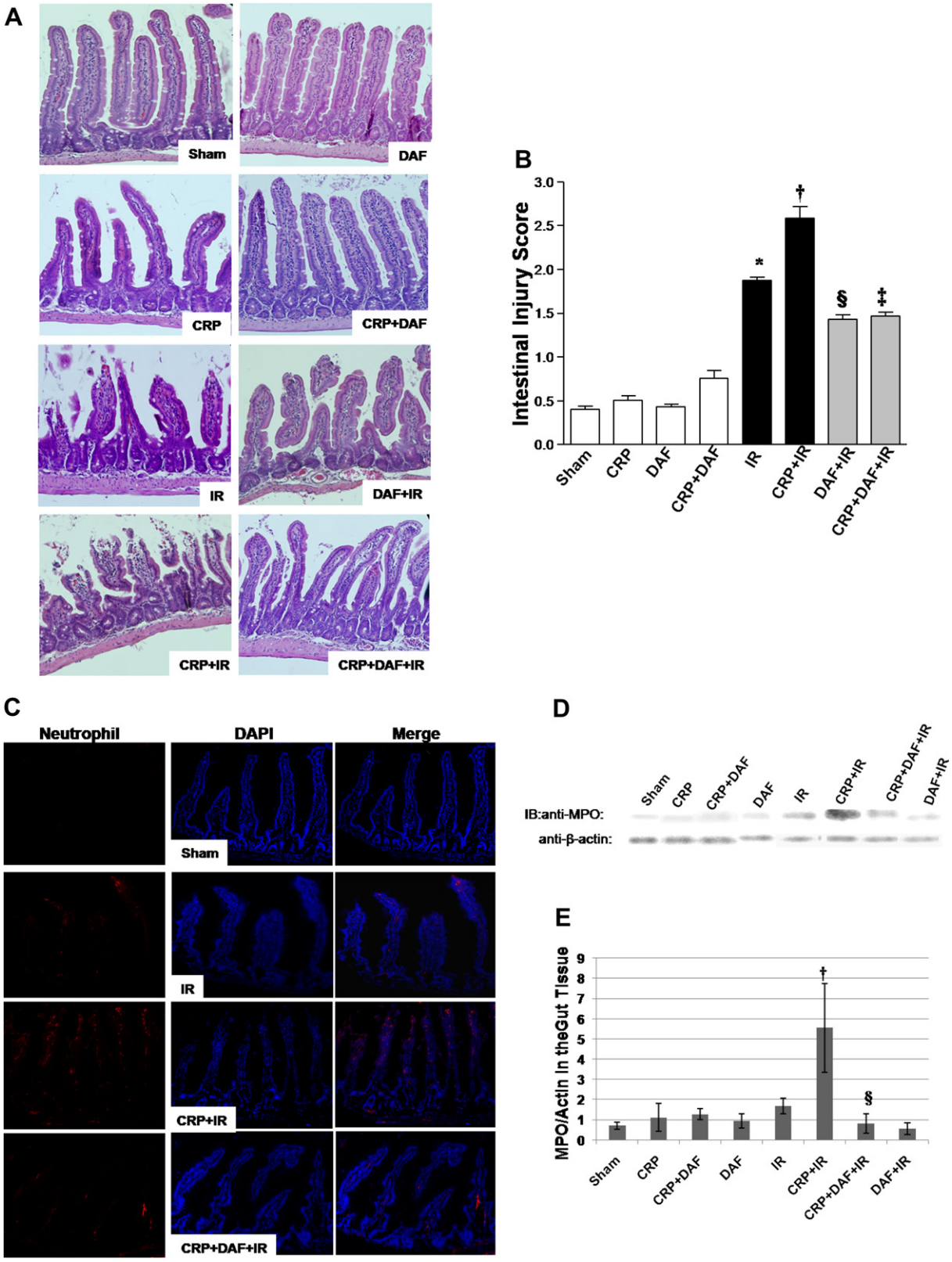

FIG. 1. DAF treatment mitigates CRP-amplified local intestinal IR injury. (A) Representative H and E stained slides from the small intestine of mice ($n = 5$–8) subjected to IR or Sham procedure and pretreated with TBS, CRP (500 μg/mouse), DAF (2 μg/mouse) were visualized and pictures were captured under a light microscope. Original magnification: ×200. (B) H and E-stained intestinal sections from various treatment groups were scored for intestinal mucosal damage as described in the Materials and Methods section. Group data were compared using one-way ANOVA followed by the Tukey's multiple comparison test with P values of $P < 0.05$ considered as significant. ANOVA: $P < 0.0001$; Tukey:

that in the Sham group (IR *versus* Sham: 2.49 ± 0.73 *versus* 0.37 ± 0.10, $P < 0.001$). CRP-pretreated animals subjected to mesenteric IR displayed more serious pulmonary injury with alveolar fibrin deposition, hemorrhage, and widespread edema, neutrophil infiltration, atelectasis, disruption of alveoli than in animals only subjected to mesenteric IR. The injury score was significantly elevated in the CRP+IR group compared with the IR group (4.17 ± 0.55 and 2.49 ± 0.73, $P < 0.001$). The lung tissue in DAF-treated animals had a reduction in these morphologic changes compared with the mesenteric IR and the CRP+IR groups. The damage scores were significantly reduced in the DAF+IR and CRP+DAF+IR groups compared with the IR and CRP+IR groups ($P < 0.001$ and $P < 0.001$, respectively). DAF not only had protective effects on local intestinal injury, but also played an effective role in protecting against CRP-enhanced acute pulmonary injury associated with mesenteric ischemia-reperfusion.

DAF Decreases PMN Infiltration in Injured Local and Remote Tissues

It has been suggested that neutrophil infiltration mediates local tissue damage in response to mesenteric IR. We reported that human CRP induced marked myeloperoxidase (MPO) activity in local damaged intestinal tissue in mice subjected to mesenteric IR [15]. To determine whether the protective effect of DAF on CRP-augmented IR injury was associated with decreased neutrophil infiltration, the neutrophils in injured areas were assessed using an anti-neutrophil antibody. Infiltrated neutrophils in frozen intestinal sections were not detected in the Sham group but were detected in mice subjected to mesenteric IR and clearly increased in the CRP+IR group. DAF injection prior to reperfusion, dramatically down-regulated the infiltration of neutrophils in the CRP+DAF+IR group compared with those in the CRP+IR group (Figs. 1C and 2C). This finding demonstrates that human DAF has a role in attenuating leukocyte recruitment in local and remote organs during acute inflammation following mesenteric IR, and that DAF reduces tissue damage associated with down-regulation of neutrophil activation.

DAF Reduces MPO Release in Damaged Tissues After Mesenteric IR

Acute tissue inflammation in this model was also evident from the MPO evaluation with western blotting. Consistent with increased neutrophil infiltration in damaged tissues, increased tissue MPO release was detected 2 h after reperfusion in the IR animals compared with the Sham group (Figs. 1D, E and 2D, E). Animals subjected to IR and pretreated with CRP (given at 180 min prior to reperfusion) exhibited significant increases in MPO deposition in the intestine and lungs. Administration of DAF 5 min prior to reperfusion significantly decreased MPO expression in the CRP+DAF+IR group compared with mice in the CRP+IR group. However, CRP, DAF alone, or CRP+DAF did not show obvious effect on MPO release in intestinal tissues compared with the Sham group.

DAF Reduced Deposition of C3 in Injured Tissues from Mice in the IR and IR + CRP Groups

Mesenteric IR results in C3 deposition on the villi [24]. To investigate whether DAF reduces complement deposition in CRP-enhanced intestinal injury, we performed immunohistochemical staining for C3. Our findings revealed significant accumulation of C3 in mice from the IR and CRP+IR groups while Sham animals did not show detectable levels of C3 in tissues. C3 accumulation in intestinal sections from the CRP+IR group was significantly greater in comparison to animals subjected to IR alone. C3 deposition in the villi was markedly reduced in DAF-treated mice subjected to mesenteric IR and pretreated with CRP (Fig. 3A). Thus, the treatment with human DAF inhibited complement activation and prevented C3 deposition in CRP-amplified mesenteric IR injury in mice.

Deposition of C3 in the lungs of mice subjected to mesenteric IR and CRP+IR was also observed. The specific C3 fluorescent signal was not as strong as that in local intestinal injury. Treatment with DAF significantly reduced CRP-enhanced intensity of C3 signal in the lung tissue following intestinal IR (Fig. 4A).

DAF Inhibits Production of Activated C5a in CRP-Accelerated Tissue Injury

C5a is an anaphylatoxin that has been identified as a major complement factor responsible for induction of the reperfusion-associated inflammatory response. In the present study, C5a accumulation was detected in local and remote injured tissues from the IR groups. In addition, a significant increase in C5a levels was detected in the CRP+IR treated animals compared with the IR and Sham controls (Fig. 3B, C and Fig. 4B, C). Administration of DAF resulted in a significant reduction of C5a deposition in the intestine and lung tissues in CRP + IR treated animals. These results indicate that a low dose

*$P < 0.001$ *versus* Sham; †$P < 0.001$ *versus* Sham, CRP and IR; §$P < 0.05$ *versus* IR; ‡$P < 0.001$ *versus* CRP+IR. (C) Neutrophil infiltration was evaluated by probing with anti-neutrophil antibody in frozen intestinal sections (original magnification: ×200). (D) MPO was detected by Western blot in gut tissues. (E) The bands of MPO were quantitated densitometrically and normalized to β-actin. †$P < 0.001$ *versus* Sham, <0.05 *versus* IR; §$P < 0.01$ *versus* CRP+IR (one-way ANOVA; Tukey post test; $n = 3 \sim 5$).

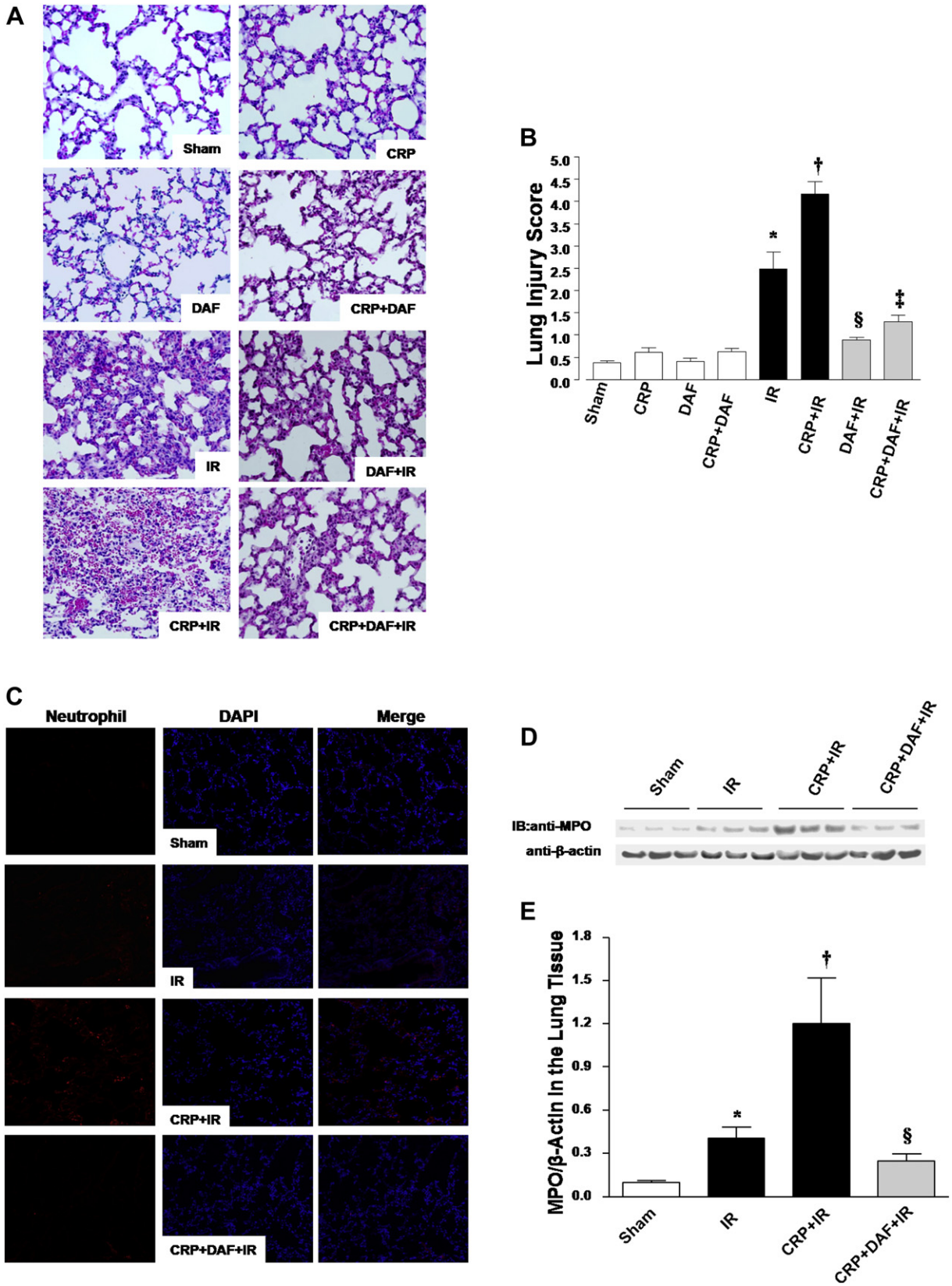

FIG. 2. DAF ameliorates detrimental effects of CRP in the lung tissue following mesenteric IR. (A) Representative H and E stained paraffin sections of the lung tissue were subjected to the same treatments as shown in Figure 1A (magnification: ×400). (B) Lung injury scores from each group were determined based on a criterion as shown in the Materials and Methods section. Group data were compared using one-way ANOVA followed by the Tukey's multiple comparison test, with $P < 0.05$ considered as significant. ANOVA: $P < 0.0001$; Tukey: $^*P < 0.001$ *versus* Sham;

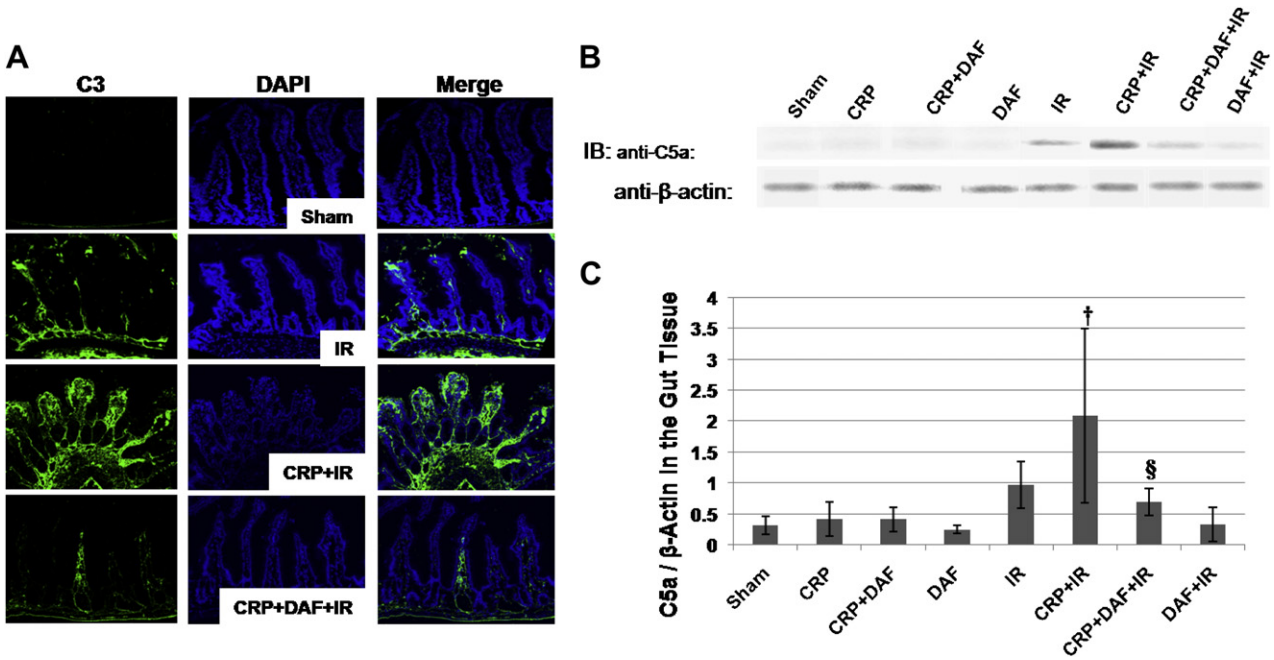

FIG. 3. DAF attenuates CRP-augmented C3 and C5a deposition in the IR-injured gut tissue. (A) Frozen sections of the small intestinal tissue were stained with anti-C3 antibody. The stained slides were observed and images were recorded under a confocal microscope equipped with a digital camera. Representative data from 5 to 8 mice were shown. Original magnification: ×200. (B) C5a of intestinal tissues was detected the western blot using anti-mouse C5a antibody. (C) The bands of C5a were quantified densitometrically and normalized to β-actin. $^{\dagger}P < 0.01$ *versus* Sham, $P < 0.05$ *versus* IR; $^{\S}P < 0.01$ *versus* CRP+IR (one-way ANOVA; Tukey post test; $n = 3 \sim 8$).

of DAF (2 μg/mouse) administration effectively attenuated the generation of C5a in damaged tissues, whereas injury was enhanced by human CRP. There was no clear difference in the C5a deposition in the gut among Sham, CRP, DAF, and CRP+DAF groups.

DAF Prevents the Formation of C5b-9 in Mesenteric IR Injury

Intestinal ischemia-reperfusion injury is mediated by and dependent on the membrane attack complex (MAC, C5b-9) [25]. In our study, DAF treatment led to decreased formation of the terminal complement component, a membrane attack complex, in CRP-augmented both local and remote injury as was demonstrated by immuno-fluorescence staining (Fig. 4D and Fig. 5A). MAC was not detected in the Sham group or CRP-treated group (data not shown). The MAC signal was stronger in the CRP+IR than IR group. CRP and MAC were co-localized in luminal membrane and surface of damaged villi of intestine or on alveolar surface and vascular endothelium in damaged lung tissue. The co-localization of CRP and MAC implicate the association and/or interaction of human CRP and murine MAC in local mesenteric IR and remote lung injury.

Western blot data revealed that C9, a component of MAC and usually considered as a representative of MAC, was significantly decreased in local gut and remote lung tissues of animals undergoing mesenteric IR and pretreated with both CRP and DAF than in the CRP +IR group (Fig. 4E and F and Fig. 5B and C). This observation further confirms that DAF reduces the MAC formation in CRP-enhanced mesenteric IR injury. No difference in C5b-9 expression in the gut was observed in Sham, CRP, DAF, or CRP+DAF animals.

DAF Suppresses Production of IL-6 in Injured Tissues Following Intestinal IR

A report indicated that intestinal IR stimulates mRNA expression of pro-inflammatory cytokines in local and remote organs in dogs [26]. Previously, we found that CRP increases gene expression of a number of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in the intestinal tissue after mesenteric IR (30—120 min) [15]. We asked whether the treatment with CRP enhances expression and deposition of pro-inflammatory cytokines in injured tissue or results in the systemic response in mice undergoing intestinal IR. We

$^{\dagger}P < 0.001$ *versus* Sham, CRP and IR; $^{\S}P < 0.001$ *versus* IR; $^{\ddagger}P < 0.001$ *versus* CRP+IR ($n = 4$–6). (C) Neutrophil infiltration in the lung tissue was determined using an anti-neutrophil antibody conjugated to fluorescence (original magnification: ×200). (D) MPO expression in lung tissue was detected by the western blot. (E) The bands of MPO were quantitated densitometrically and normalized to β-actin. $^{*}P < 0.05$ *versus* Sham; $^{\dagger}P < 0.05$ *versus* IR; $^{\S}P < 0.05$ *versus* CRP+IR (two-tailed *t*-test; $n = 3$).

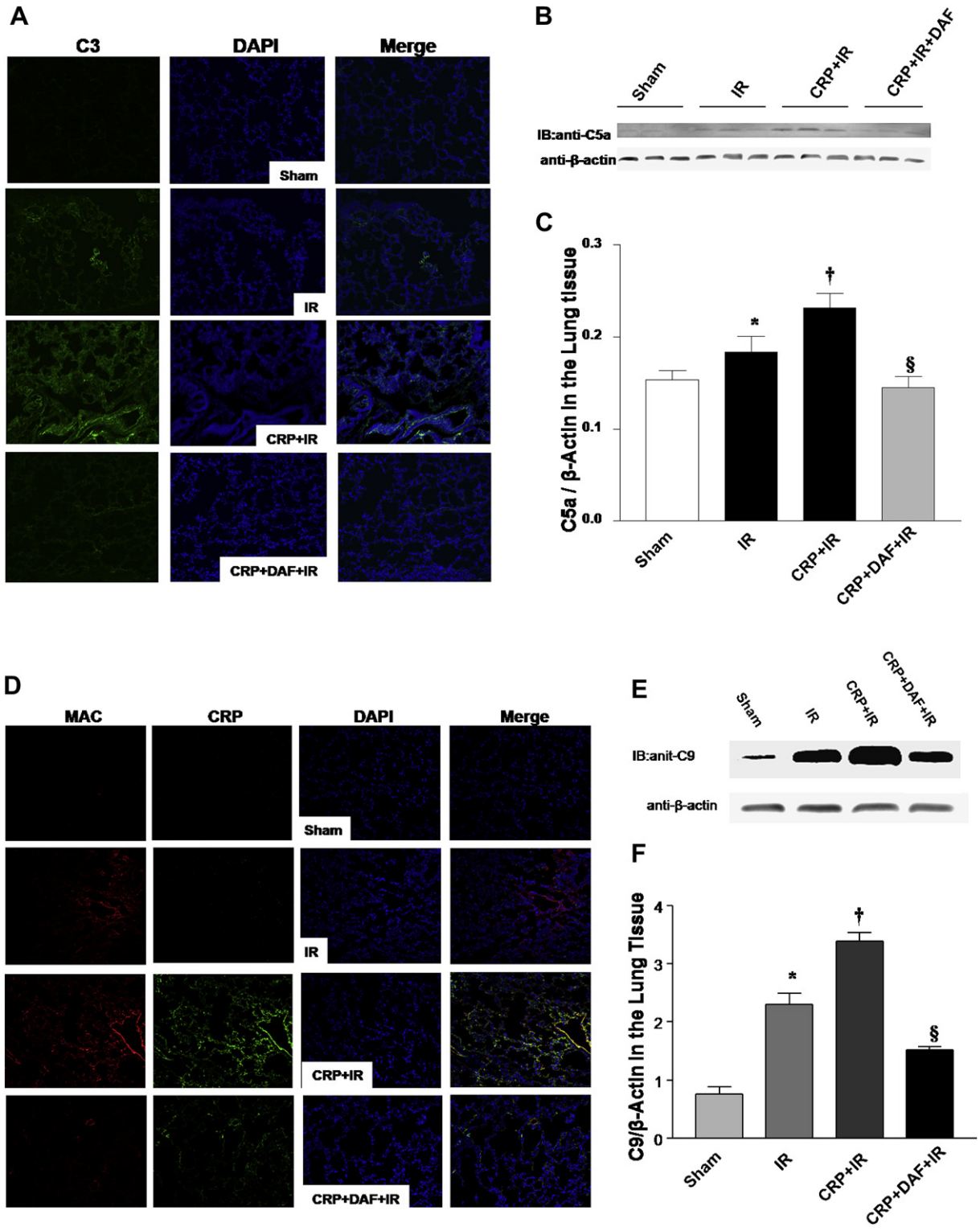

FIG. 4. DAF reduces CRP-exacerbated C3, C5a, and MAC deposition in the lung tissue following mesenteric IR. (A) C3 deposition in the lung tissue was assessed by immunofluorescent staining and confocal microscopy. Each image is representative of three experiments. Original magnification: ×200. (B) Deposition of C5a was analyzed by immunobloting the lysates from lung tissue. (C) The bands were scanned and the density related to β-actin was calculated. *$P < 0.05$ *versus* Sham; $^{\dagger}P < 0.05$ *versus* Sham; $^{\S}P = 0.01$ *versus* CRP+IR (two-tailed *t*-test; $n = 3$). (D) MAC deposition was detected by immunolabeling frozen section of lung tissues with anti-MAC and anti-CRP antibodies, and representative micro-photographs from three mice were shown at ×200 magnification. (E) C9 was analyzed in the lung tissue by the Western blot and each band was representative of three experiments. (F) The C9 bands were quantitated densitometrically and the ration was calculated to β-actin. *$P < 0.001$ *versus* Sham; $^{\dagger}P < 0.001$ *versus* Sham, <0.01 *versus* IR; $^{\S}P < 0.05$ *versus* IR, <0.001 *versus* CRP+IR (one-way ANOVA; Tukey post test, $n = 3$).

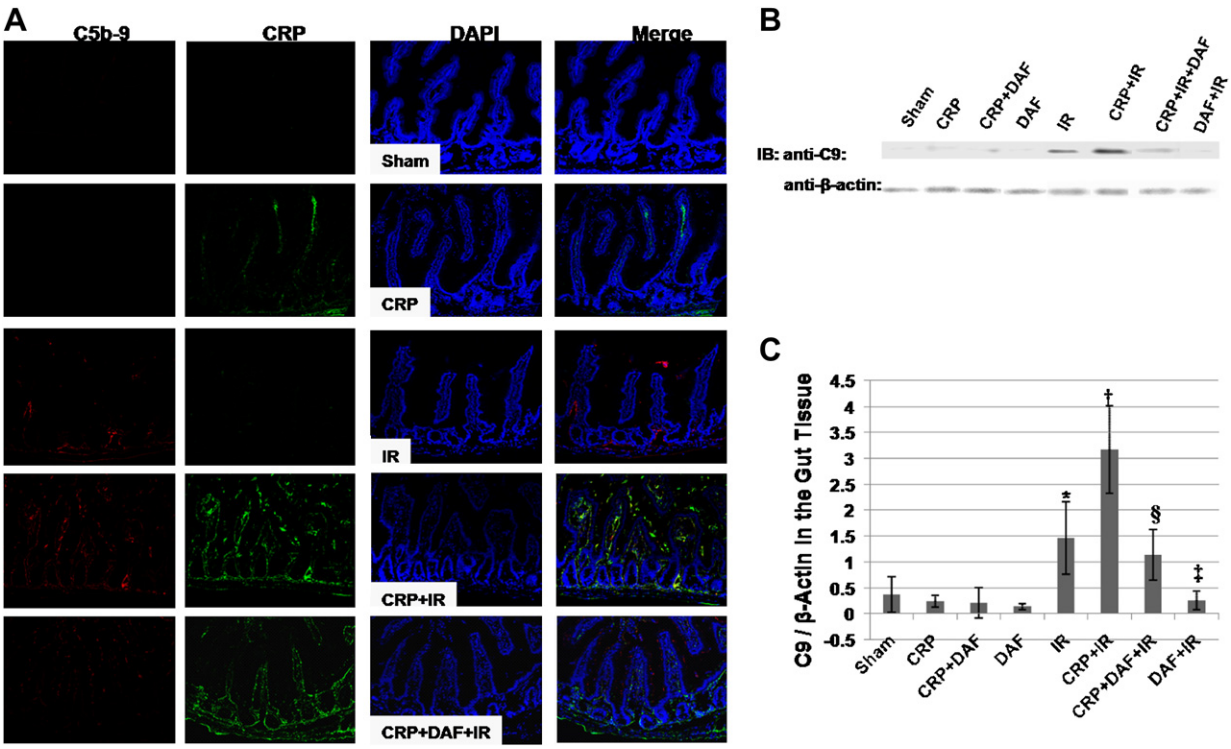

FIG. 5. DAF weakens CRP-strengthened deposition of C9 in the IR-damaged intestinal tissue. (A) Frozen sections from the gut tissues were stained with anti-MAC and anti-CRP antibodies, then stained slides were visualized by confocal microscopy. Image is representative of three sections from at least three different mice. Original magnification: ×200. (B) Gut tissue was lysed and immunoblotted with anti-C9 and anti-β-actin antibodies to detected C9 deposition. (C), C9 was quantified by densitometry and the ratio to β-actin was calculated for each sample. Cumulated data from three independent experiments are shown. * $P < 0.01$ *versus* Sham; $^{\dagger}P < 0.001$ versus Sham and IR; $^{\S}P < 0.001$ *versus* CRP+IR; $^{\ddagger}P < 0.05$ *versus* IR (one-way ANOVA; Tukey post test, $n = 3 \sim 8$).

also asked whether DAF, as a G-coupled protein, can reduce inflammatory cytokines during mesenteric IR in mice. We used ELISA to determine IL-6 levels in murine sera. The serum concentration of IL-6 was significantly increased in the IR group (125.65 ± 31.45 pg/mL *versus* Sham 31.53 ± 21.83 pg/mL, $P = 0.0034$) and the CRP+IR group (148.98 ± 41.43 pg/mL *versus* CRP+Sham 71.95 ± 19.78 pg/mL, $P = 0.0017$). This indicates that IR injury triggers IL-6 expression and release, leading to its elevated blood concentration. There was no significant difference between the CRP and IR group, the CRP and Sham group, the CRP+DAF and Sham group in the serum IL-6 level. Administration of DAF (2 μg/mouse) markedly down-regulated serum IL-6 levels in the DAF+IR group (DAF+IR 75.97 ± 29.09 *versus* IR 125.65 ± 31.45, $P < 0.05$), but DAF did not significantly decrease serum IL-6 in the CRP+DAF+IR group (Fig. 6A).

Deposition of IL-6 in damaged tissue following mesenteric IR has not been reported before. We found that IL-6 was expressed and deposited 2 h after reperfusion, not only locally in the intestinal tissue, but also in remote lung tissue. IL-6 was not detected in these tissues of the Sham group. As shown in Fig. 6B and C, human CRP enhanced IL-6 deposition and co-localized with murine IL-6 in the intestinal and lung tissues in the CRP+IR group. The treatment with DAF remarkably reduced deposition of IL-6 in the intestinal and lung tissues in the CRP+DAF+IR group. Therefore, these data indicate that IL-6 is induced in the early phase of mesenteric IR and might have participated in the IR-initiated acute inflammatory reaction. DAF, a complement inhibitor, plays a protective role against CRP-augmented mesenteric IR injury, at least partially, by the inhibition of local and systemic IL-6 activity.

DISCUSSION

In this study, we have demonstrated that DAF clearly reduces CRP-enhanced intestinal and lung injury following acute mesenteric IR in mice. In particular, a direct or an indirect effect of DAF on inhibition of the complement activation, suppression of the neutrophil infiltration, and reduction of the IL-6 production plays a key role in its protective actions on CRP-enhanced murine tissue injury of mesenteric IR. This is the first report of beneficial effects of DAF on CRP-potentiated local and systemic injury.

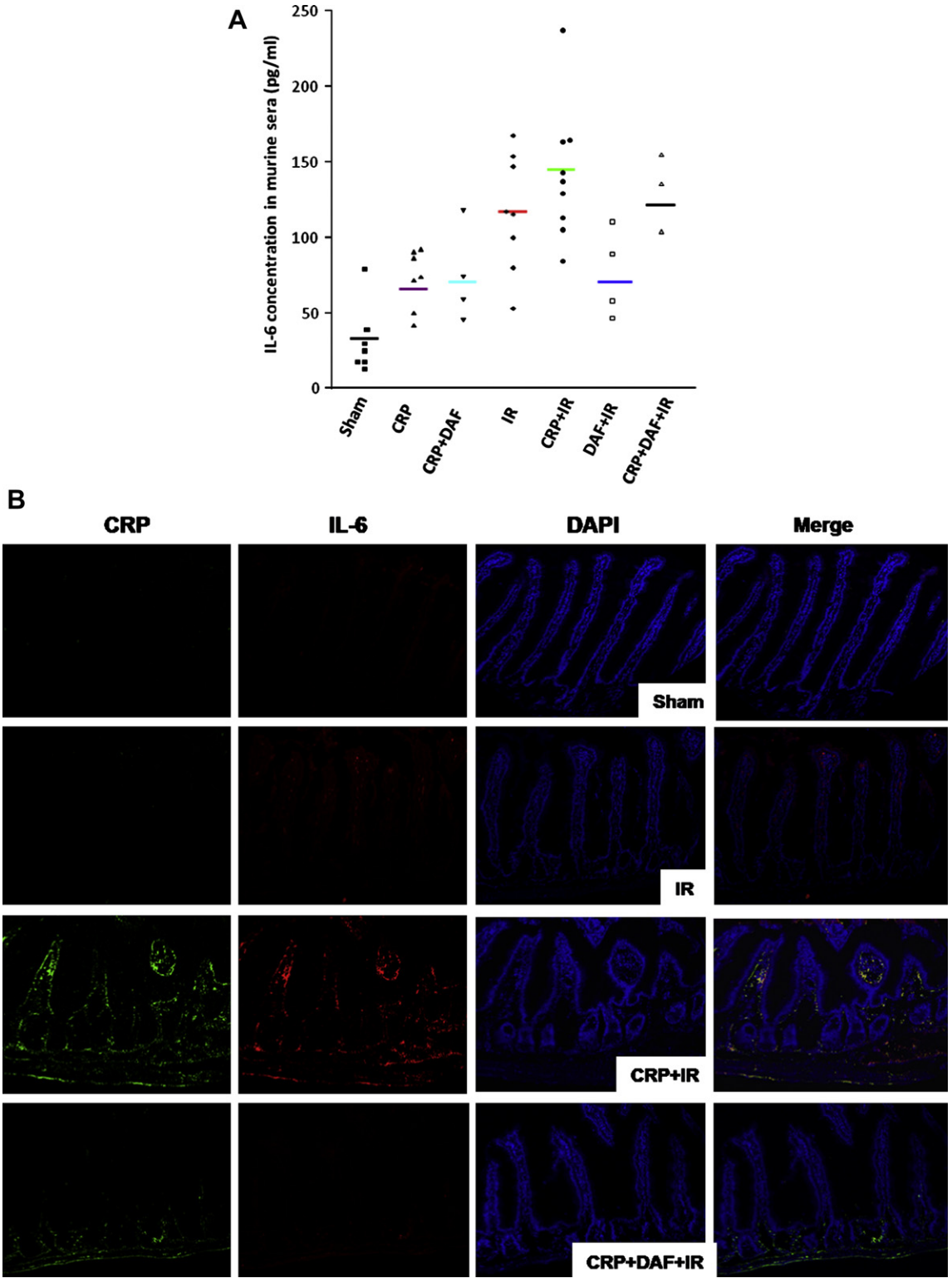

FIG. 6. DAF decreases production of IL-6 locally and systemically after mesenteric IR. (A) Serum IL-6 concentration was determined by ELISA. Red bar indicates a significant difference *versus* Sham, $P < 0.05$; green bar displays significant difference *versus* Sham, $P < 0.001$, *versus* CRP, $P < 0.01$, *versus* CRP+DAF, $P < 0.05$; blue bar indicates significant difference *versus* IR, CRP+IR, $P < 0.05$ (one-way ANOVA; Tukey post test). (B) Expression of IL-6 in the intestinal tissue was measured by immunostaining with anti-IL-6 antibody. Presented data is from a representative experiment of three separate studies. Original magnification: ×200. (C) Production of IL-6 in lung tissue after intestinal IR was determined by immunofluorescent labeling using anti-IL-6 antibody. Each image is representative of three separate experiments. Original magnification: ×600.

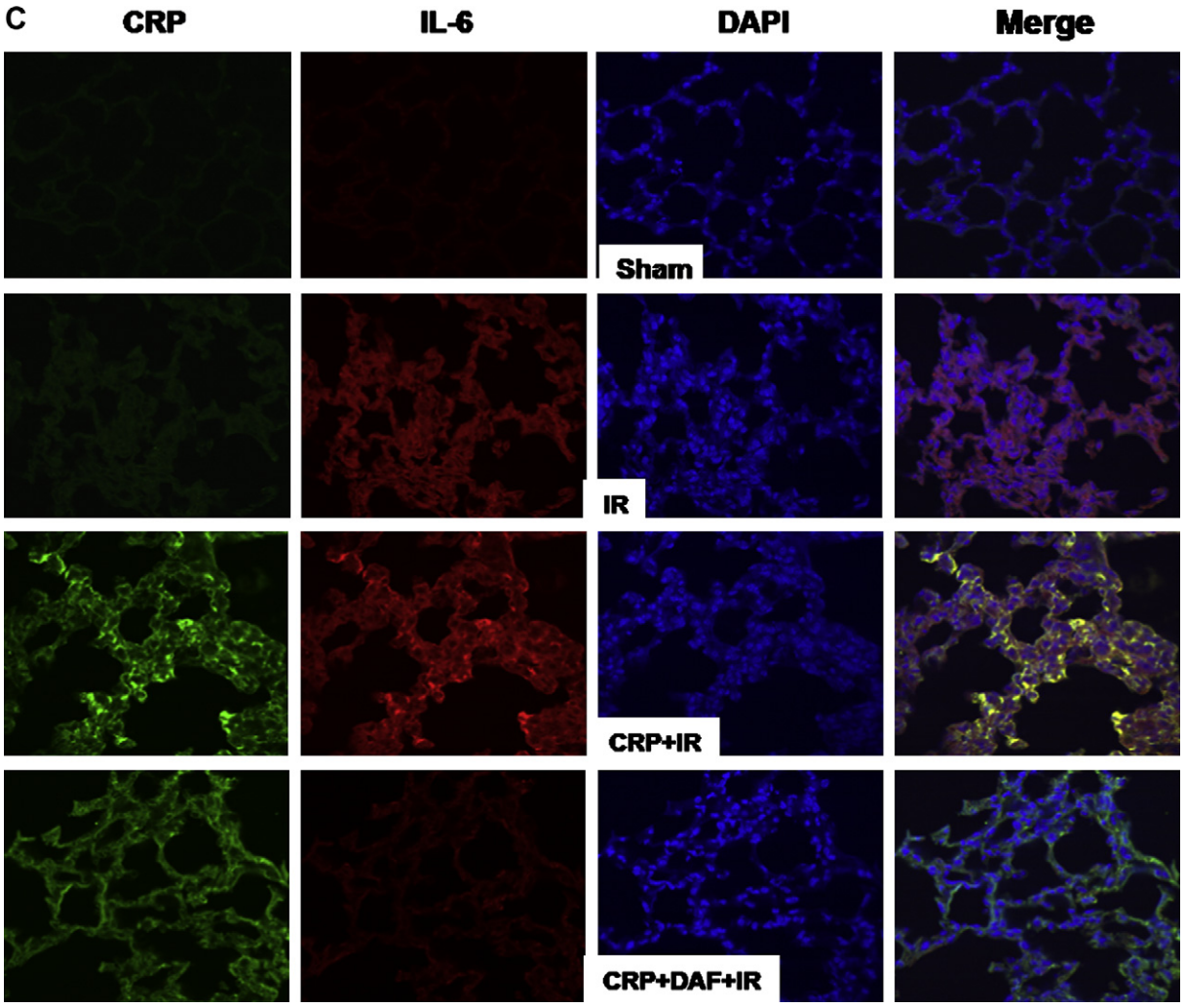

FIG. 6. (*continued*).

CRP is known to initiate the complement classic pathway [27] and it is the key molecule that boosts the immune response [28]. Complement activation has a critical role in the pathologic alterations following mesenteric IR in animals [4]. Further, the endogenous and exogenous CRP takes part in the pathogenesis and potentiates intestinal IR injury [14, 15]. We have shown that CRP enhanced mesenteric IR in a complement-dependent manner. This synergistic effect of CRP on tissue damage can be prevented by the complement depletion with cobra venom factor [15]. Studies reported that the inhibition of complement activation using membrane complement regulatory proteins, including endogenous [17] and exogenous DAF [18], provided us with an important insight into protection against the potentiating effect of CRP on IR injury.

We selected the therapeutic dose of DAF as 2 μg per mouse in this study as per our previous report [18]. In this study, human DAF concentration in murine serum was not monitored since it has been reported that human DAF has a very short half-life in the circulation, and that the majority of DAF rapidly binds to and deposits on the cell membranes in local and remote tissues 10 min after administration [18]. Immunofluorescent staining showed that DAF deposition still remained bound to the epithelial and endothelial surface in the intestinal and to the endothelial surface in the lung tissue 2 h after injection in all treated mice (unpublished results). DAF is more stable and active on membrane-associated complement complexes [29, 30]. Once bound and incorporated, it seems to inhibit activation and amplification of complement cascade, which leads to local and systemic damage. The Western blot indicated that administration with as low as 2 μg of DAF effectively reduced C5a formation in local intestinal and remote lung tissue following mesenteric IR in mice pretreated with CRP. DAF significantly attenuated CRP-augmented morphologic injury and neutrophil infiltration in both intestinal and lung tissues. This was

consistent with an observed decrease of C5a and C5b-9 deposition in the tissues.

Mesenteric IR causes local and systemic inflammatory derangements, including release of pro-inflammatory cytokines, and is associated with complement activation. Reports indicate the intestine is a source of cytokine production following intestinal IR injury [31, 32]. In the present study, IL-6 release was found not only in local intestinal tissue, but also in remote lung tissue. The secretion of IL-6 from damaged intestinal and lung tissue may contribute to the increase of the systemic level of IL-6 in mice subjected to mesenteric IR. We did not evaluate the IL-6 production in other tissues and, therefore, we do not know the possible contribution of IL-6 release from these tissues.

The exact mechanism involved in CRP-induced IL-6 secretion remains unclear. Generation of IL-6 appeared to require the involvement of Fcγ receptors, such as CD16 and CD32, on inflammatory cells [33]. CRP has been shown to bind to Fcγ receptors and subsequently lead to activation of PI3 K/Akt, ERK, and NF- κB pathways [33]. Activation of these signal pathways have been known to up-regulate cytokine production [33]. In this study, the pretreatment of animals with human CRP prior to ischemia obviously enhances the IL-6 release in both intestinal and lung tissues. This effect of CRP on IL-6 release could be explained by CRP binding to its ligands on the surface of activated macrophages and neutrophils in injured tissues. Indeed, human CRP co-localization with murine CD32 rather than CD16 was observed on the surface of inflammatory cells in IR-damaged tissues (unpublished results). It implies that human CRP may interact with murine CRP ligand undergoing IR *in vivo*, which initiates or enhances activation of PI3 K/Akt, ERK, and NF- κB pathways, thereby potentiating IL-6 production in mesenteric IR injury.

The fact that DAF can effectively reduce the CRP-enhanced IL-6 release from injured tissues is the most important finding in this study. DAF inhibits C3 and C5 convertases in both the classic and the alternative complement pathways and, thus, suppresses the generation of C3a and C5a. These anaphylatoxins, especially C5a, can induce synthesis and release of pro-inflammatory cytokines by leukocytes [34]. DAF-deficient mice are more susceptible to complement-mediated inflammatory injury [17, 35], and produce extraordinary serum levels of IL-6 upon stimulation with lipopolysaccharide compared with wild-type animals [35]. Our data showed that C5a was significantly decreased in damaged gut and lung tissues in mice pretreated with CRP and then treated with DAF prior to intestinal reperfusion than in control, which are mice with mesenteric IR only pretreated with CRP. Thus, the attenuation of IL-6 expression by DAF in damaged tissues could be, at least partly, attributed to the inhibition of an anaphylatoxin, C5a.

One of the serious consequences of mesenteric IR is multiple organ failure, which is also referred to as secondary organ injury [36]. Complement activation leads to the generation of anaphylatoxin C3a and C5a, which contribute to the development of remote organ injury [18, 37]. Other studies reported that intestinal IR-induced pro-inflammatory cytokines and chemokines exert their effects *via* a direct toxic action on target cells in distant organs [38, 39]. Complement activation results in the release of chemoattractants C3a and C5a, which can be released directly or indirectly through the activation of endothelial cells expressing other various chemokines and adhesion molecules and subsequently activate neutrophils [35, 40].

A previous study suggested that the membrane attack complex (C5b-9) also solely mediated neutrophil infiltration following mesenteric IR injury in mice [18]. The influx of neutrophils into IR tissue can cause degranulation and superoxide production, which damages tissue and subsequently amplifies the neutrophil response. The accurate mechanisms regulating remote lung injury are not fully explained but probably involve complement activation, cytokine/chemokine generation, leukocyte infiltration, and release of reactive oxygen species (ROS) and proteases into remote tissue. In this study, DAF inhibited C5a formation, IL-6 expression, accumulation of neutrophils, and release of MPO in both intestinal and lung tissue in mice with CRP-enhanced mesenteric IR injury. By inhibition of C5a production and MAC formation, DAF blocks the downstream signaling of C5a/C5a receptor (C5aR) and MAC. This infers that complement-mediated inflammation can be modulated at C5a/C5aR level, resulting in prevention of local and remote organ injury. Our study suggests that DAF may be useful for the treatment of acute intestinal injury associated with bowel obstruction, necrotic enterocolitis, repair of abdominal aortic aneurysms, and acute mesenteric ischemia.

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