ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Clinical Immunology 108 (2003) 263–273

CLINICAL IMMUNOLOGY

www.elsevier.com/locate/yclim

C5a causes limited, polymorphonuclear cell-independent, mesenteric ischemia/reperfusion-induced injury☆,☆☆

Sherry D. Fleming,[a,b,*] Dimitrios Mastellos,[c] Georg Karpel-Massler,[a] Terez Shea-Donohue,[b,d] John D. Lambris,[c] and George C. Tsokos[a,b]

[a] *Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500, USA*
[b] *Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA*
[c] *Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*
[d] *Beltsville Human Nutrition Research Center, U.S. USDA-ARS, Beltsville, Beltsville, MD 20705, USA*

Received 2 May 2003; accepted with revision 3 June 2003

Abstract

C5 is critical in the development of local mucosal damage and inflammation as well as in the development of remote organ injury after mesenteric ischemia/reperfusion (IR). To define the role of C5a in tissue injury, we treated wild-type mice with a cyclic hexapeptide C5a receptor antagonist (C5aRa) and administered recombinant C5a to C5 deficient ($C5^{-/-}$) mice subjected to mesenteric IR. We demonstrate that at 2-h postreperfusion, C5a administered to C5−/− mice during IR induces limited intestinal mucosal injury but failed to cause remote lung injury despite the fact that it upregulated adhesion molecule expression. C5aRa treatment of C5+/+ mice undergoing IR limited local injury and prevented distant organ injury. We conclude that although C5a can trigger certain components of the IR induced injury, other mediators such as C5b-9 and local factors are needed for the complete expression of IR tissue damage.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Complement; Neutrophils; Inflammation; Rodent; Mucosa; Adhesion molecules

Introduction

Ischemia/reperfusion (IR)[1] results in local intestinal inflammation and damage, and is associated with systemic inflammatory responses in remote organs including the lungs [1]. The reperfusion phase that follows ischemia is responsible for the observed local and remote organ damage, because multiple inflammatory mediators are generated and distributed to tissues by the circulating blood. These factors include reactive oxygen species, nitric oxide, eicosanoids, cytokines, acute phase proteins, and complement activation products (reviewed in [1]).

Reperfusion after mesenteric ischemia causes local inflammation characterized by complement activation and deposition, neutrophil infiltration, and eicosanoid generation that coincides with mucosal injury [2,3]. This inflammatory response of the intestine sets up a "priming" bed where systemic PMN are activated by the intestine-derived mediators including platelet activating factor and interleukin-6 [4,5]. Activated peripheral blood neutrophils are believed to be the main contributors to the development of the systemic inflammatory response and ultimately multiple organ failure [1].

Activation of C5 leads to the production of a potent

☆The opinions contained herein are the private ones of the authors and are not to be construed as official policy or reflecting the views of the Department of Defense.

☆☆These studies were performed while SDF held a National Research Council Fellowship and were supported by STOC of the Medical Research and Material Command and National Institutes of Health grants NIH R0-1 GM62134 (JDL) and AI49316 (TSD).

* Corresponding author. Walter Reed Army Institute of Research, Room 1A32, Building 503, Robert Grant Road, Silver Spring, MD 20910-7500. Fax: +1-301-319-9133.

E-mail address: sfleming@usuhs.mil (S.D. Fleming).

[1] Abbreviations used: IR, ischemia/reperfusion; PMN, polymorphoneutrophil; C5aRa, C5a receptor antagonist; LTB4, leukotriene B4; PGE2, prostaglandins E2; BAL, bronchoalveolar lavage; mC5a, mouse C5a sequence.

1521-6616/03/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S1521-6616(03)00160-8

anaphylotoxin, C5a, along with C5b, the initiator of the membrane attack complex. Inhibition of C5 activation by an anti-C5 antibody administered to wild-type mice subjected to mesenteric IR prevents C5a generation, PMN infiltration, and deposition of the terminal complement complex on the damaged tissues in a manner similar to that observed in C5 deficient (C5−/−) mice [6–11]. These studies however, do not distinguish between the actions of C5a and C5b-9 terminal complex.

C5a, a small, glycosylated peptide, is a potent chemoattractant for PMN, monocytes, and T cells (reviewed in [12,13]). In vitro studies have shown that C5a induces degranulation and respiratory burst, increases adhesion molecule expression, and delays apoptosis in PMN [14–16]. In addition, in vivo studies using anti-C5a antibodies have indicated that C5a alters vascular permeability and neutrophil activation during cardiopulmonary bypass [17], hind limb IR [18], sepsis [19–22], and inflammatory lung injury [23]. C5a receptors (C5aR) are expressed on the surface of intestinal cells under inflammatory conditions, as well as on bronchial epithelial cells [24]. Blockade of these receptors using C5aR antagonists (C5aRa) indicates a role for C5a in systemic activation of neutrophils in multiple animal models [25–28].

To distinguish the role of C5a from that of C5b-9 on local and remote tissue injury, we administered a cyclic hexapeptide C5a receptor antagonist (C5aRa) to wild-type type mice to inhibit the action of C5a during mesenteric IR and administered C5a to $C5^{-/-}$ mice subjected to mesenteric IR. These experiments showed that during IR, C5a is sufficient to induce limited local damage and eicosanoid production but not systemic PMN activation. In addition, C5a administered intraveneously during IR can induce VCAM expression on remote organs such as the lung without inducing increased vascular permeability.

Material and methods

Animal preparation

Adult, male C57B1/6 mice (NCI, Bethesda, MD), C5 deficient mice, B10.D2-H2D H2-T18c.HC1/oSJN, ($C5^{-/-}$) (Jackson Laboratory, Bar Harbor, ME), and the wild-type controls, B10.D2-H2D H2-T18c.HC0/nSJN ($C5^{+/+}$) (Jackson Laboratory, Bar Harbor, ME) were obtained and prepared for surgery after a 7-day acclimation period in the Walter Reed Army Institute for Research animal facility. Mice were anesthetized with ketamine (16 mg/kg) and xylazine (8 mg/kg) administered ip. All procedures were performed with the animals breathing spontaneously and body temperature maintained at 37°C using a water-circulating heating pad. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition. 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.

Cloning and bacterial expression of mouse C5a

Total RNA was prepared from mouse liver using the RNeasy isolation kit (Qiagen, Chatsworth, CA) according to manufacturer's instructions. The mouse C5a sequence (mC5a) was amplified from total liver RNA by RT-PCR (One-step RT-PCR kit, Qiagen) using the following primers: 5′-primer, 5′-CCTGGATCCAATCTGCATCTCCTA-AGG-3′; 3′-primer, 5′-TTCAAGCTTTCACCTTCCCAGTT-GGACAGG-3′. mC5a was subsequently subcloned into the pQE-30 expression vector (Qiagen) at the *Bam*HI and *Hin*dIII restriction sites. Expression of mC5a was induced in M15[pREP4] *Escherichia coli* by the addition of 1 mM IPTG, at 37 °C for 4 h. Bacterial cell pellets were treated with 8 M urea, 10 mM β-mercaptoethanol, 10 mm imidazole, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, 0.3 M NaCl, pH 8.0, and recombinant mC5a was subsequently purified using Ni-NTA metal-chelating affinity chromatography (Qiagen). mC5a was eluted from the column with 1 ml fractions of 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, 0.3 M NaCl, at pH 4.5. Purified C5a was refolded by dialysis overnight in 1000X volume of 0.1 M Tris-Cl pH 8.0, 2 mM reduced glutathione, 0.2 mM oxidized glutathione, 0.005% Tween 80. The biologic activity of the refolded C5a fraction was evaluated in a Transwell chemotaxis assay (Costar) using the RAW.274 mouse macrophage cell line. The specific C5aR antagonist AcF[OpdChaWR] used in this study was synthesized as described previously [29,30].

Experimental protocol

Animals were subjected to IR as described previously [31,32]. Briefly, a midline laparotomy was performed prior to a 30-min equilibration period. The superior mesenteric artery was identified and isolated, and a small vascular clamp (Roboz Surgical Instruments, Rockville, MD) applied for 30 min. Ischemia was confirmed to the mid-jejunum by noting a color change in the intestine from pink to gray and an absence of pulsations of the mesenteric vessels distal to the clamp. Covering the bowel with surgical gauze moistened with warm 0.9% normal saline prevented dessication of the intestine. After 30 min of mesenteric ischemia, the clamp was removed under direct visualization and the intestine reperfused for 2–4 h. Five minutes prior to the start of reperfusion, 1 μg C5a and/or 25 μg C5aRa was administered iv to some animals. Sham treated animals underwent the same surgical intervention except for the superior mesenteric artery (SMA) occlusion. An IR control group underwent identical surgical intervention with an

equivalent amount of PBS. After clamp removal, reperfusion was confirmed by observing a return of the pink color of the bowel and the pulsatile flow to the SMA and its branches. Laparotomy incisions were sutured and the animals monitored during the reperfusion period, with additional ketamine and xylazine administered immediately prior to sacrifice. After sacrifice, the small intestine 10 cm distal to the gastroduodenal junction was harvested for histologic evaluation, and quantification of PMN infiltrate. There was no significant difference in the survival of the various treatment groups.

Histology and immunohistochemistry

Tissues from small intestine were promptly fixed in 10% buffered formalin phosphate, embedded in paraffin, sectioned transversely (5–7 μ), and stained with Giemsa. The mucosal injury score (SMI) was graded on a six-tiered scale similar to that defined by Chiu et al, [33]. The villus height and crypt depths of at least 10 villi from the same sections were measured using an ocular micrometer.

Additional tissue was fixed for 2 h in cold paraformaldehyde prior to transfer to PBS, paraffin embedding, and preparation of transverse sections. Nonspecific antigen binding sites were blocked by treatment with a solution of 10% rat sera in phosphate-buffered saline (PBS) for 30 min. After washing in PBS, the tissues were incubated with FITC-conjugated, granulocyte specific, GR-1 monoclonal antibody (PharMingen, San Diego, CA) for 1 h at room temperature and washed, and slides were mounted using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Other sections were incubated with goat anti-mouse VCAM overnight (Santa Cruz). The anti-VCAM sections were then incubated with a FITC-conjugated rabbit anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA). The slides were examined by fluorescent microscopy using a Leica DM RX/A fluorescent microscope (Leica Microsystems, Atlanta, GA) with SPOT diagnostic computer software (Sterling Heights, MI). The number of GR-1 positive cells/high power field were counted and averaged in three separate fields.

Eicosanoid generation

The ex vivo eicosanoid generation by the intestine was determined as described previously [31,32]. Briefly, sections of midjejunum were minced, washed, and resuspended in oxygenated 37 °C Tyrode's buffer (Sigma, St. Louis, MO). After 20 min incubation at 37 °C, the supernatants and tissues were collected and stored until assayed at −80 °C. Tissue protein was determined using a BCA protein kit (Pierce, Rockford, IL). LTB_4 and PGE_2 concentrations were determined via enzyme immunoassays (Cayman Chemical, Ann Arbor, MI) following the manufacturer's directions.

Peroxidase activity

Supernatants generated for the eicosanoid assays were also used for determination of tissue peroxidase activity using 3,3′,5,5′ tetramethylbenzedene (TMB) as described previously [33]. Briefly, supernatants were incubated with equal volumes of TMB peroxidase substrate (Kirkgaard and Perry, Inc, Gaithersburg, MD) for 45 min. Addition of 0.18 M sulfuric acid stopped the reaction and the OD_{450} was determined. Total peroxidase concentration was determined by comparing to a horseradish peroxidase standard (Sigma) and reported as picogram peroxidase activity per milligram tissue.

Capillary leak

One hour prior to sacrifice, mice were injected iv with 0.1 ml of 0.5% Evan's blue dye. Immediately after mice were sacrificed, bronchoalveolar lavage (BAL) was performed by infusing 1 ml normal saline by tracheotomy. After waiting 30 s the effluent was aspirated. The recovered fluid was centrifuged to collect the cells and the cells were counted. Optical densities (A630) of the fluid and sera were determined and the ratio calculated as a measure of in vivo pulmonary macromolecular capillary leak.

Fluorescence staining and analysis

Blood was obtained by cardiac puncture immediately prior to sacrifice. After RBC were lysed in 3 ml RBC lysis buffer (Sigma, St. Louis, HO), the peripheral blood leukocytes were centrifuged and resuspended in PBS, aliquots of cells were incubated on ice for 15 min in staining buffer (PBS containing 10% FBS, and 0.2% sodium azide), and specific antibody or isotype control antibody. Additional aliquots of cells were incubated at 37 °C in PBS and 2 mM 2′,7′-dichloro-fluorescine diacetate to determine oxidative burst as described previously [4]. After all incubations, the cells were washed with staining buffer, centrifuged, and resuspended in staining buffer prior to analysis on an ELITE flow cytometer (Coulter Electronics Inc). The log fluorescence intensity of PMN from each sample was displayed as a frequency distribution histogram. The gate used to identify the PMN population was based on the forward and side scatter of GR-1 positive cells.

Statistical analysis

Data are presented as mean ± SEM and were compared by one-way analysis of variance with post hoc analysis using the Newman-Keuls test (Graph Pad/Instat Software Inc. Philadelphia, PA). The difference between groups was considered significant when $P < 0.05$.

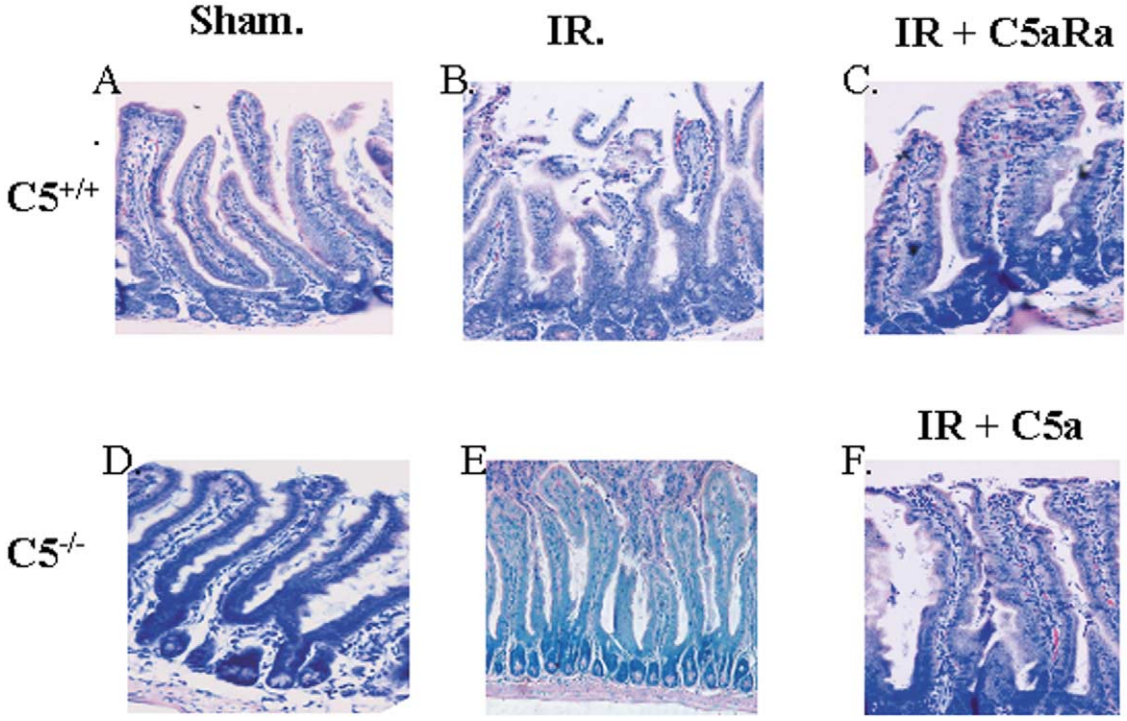

Fig. 1. Histological evidence that C5a plays a role in intestinal ischemia/reperfusion injury. Representative Giemsa-stained, formalin-fixed intestinal sections from $C5^{+/+}$ mice (A–C) or $C5^{-/-}$ mice (D–F) were subjected to sham treatment (A,D), IR (B,E), IR + C5aRa (C), or IR + C5a (F). Each figure is representative of 6–8 mice/group. All photomicrographs are 200X magnification.

Results

C5a contributes to local mesenteric IR-induced injury

Previous studies have shown that C5 activation is required for IR-induced local injury [11]. To determine whether C5a has a role in local damage, we compared the intestinal injury of C5 sufficient ($C5^{+/+}$) to that of $C5^{-/-}$ mice and $C5^{+/+}$ mice treated with C5aRa during IR (Fig. 1 and 2). Similar to previous findings, $C5^{+/+}$ mice subjected to IR displayed severe mucosal damage after 2 h reperfusion [11]. Damage was indicated macroscopically by dark red, swollen, edematous intestines. Histologically, many villi were disrupted with exposed lamina propria and hemorrhage. The overall villus height is decreased with edema in the submucosa. Mice deficient in C5 failed to express similar tissue damage. When C5aRa was administered 5 min prior to reperfusion, the intestinal damage decreased significantly when compared to $C5^{+/+}$ mice subjected to IR. It should be noted that C5aRa did not completely prevent the IR-induced injury. C5aRa injected into $C5^{-/-}$ mice subjected to IR did not have any effect on tissue histology (Fig. 2).

We next sought to determine whether C5a is directly responsible for inducing local IR-induced damage. In initial experiments we titrated the C5a dose and found that 1 μg C5a given to C5−/− mice in conjunction with IR allowed >90% survival for the duration of the 2 h reperfusion phase. C5a administered in either one single 3 μg dose or three 1 μg doses over 45 min, resulted in death of five out of six mice. Additional mice were given a total of 1 μg C5a in three separate doses with no significant difference (data not shown). Therefore, all data presented after administration of C5a represent those obtained after the administration of a single dose. Similar one-dose protocols have been used before in a rodent model of immune complex deposition-mediated acute lung injury [23,34]. When $C5^{-/-}$ mice were

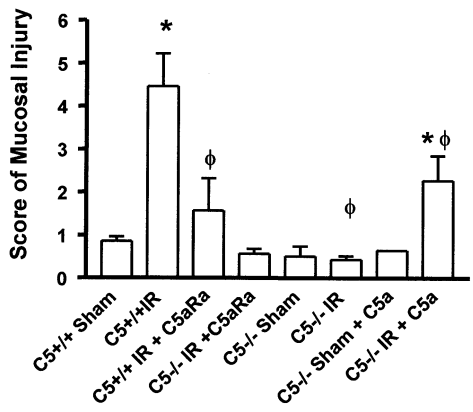

Fig. 2. Quantitative analysis of intestinal IR injury. Giemsa stained intestinal sections from each treatment group were scored for mucosal injury (0–6) as described in Materials and Methods. All measurements were obtained at 200X magnification. Each bar is the average ± SEM with 6–8 mice/group. Using ANOVA with Neuman Keuls post hoc test, * indicates significant difference from sham treatment group, $P < 0.05$. ϕ indicates significant difference from IR treatment of $C5^{+/+}$ mice $P < 0.05$.

given 1 μg C5a 5 min prior to reperfusion, a significant increase in intestinal mucosal damage was observed (Fig. 2). However, the injury was significantly less than that seen in $C5^{+/+}$ mice subjected to IR. In addition, administration of C5aRa in conjunction with C5a to C5−/− mice prevented intestinal injury (mean injury score ± SEM = 1.03 ± 0.3, $n = 7$). Therefore, C5a alone can instigate IR-induced tissue injury.

PMN infiltration does not accompany the C5a-mediated injury

It has been suggested that neutrophil infiltration mediates local tissue damage in response to IR. Therefore, we quantitated PMNs in the intestinal tissue after sham or IR treatment. As shown previously, $C5^{+/+}$ mice had a significant increase in PMNs within the intestinal tissue after 2 h reperfusion (Fig. 3A and [11]). Correlating with the injury score, PMN infiltration was decreased in $C5^{-/-}$ mice subsequent to IR. The administration of C5aRa to $C5^{+/+}$ mice prevented the IR-induced PMN infiltration. This data suggest that C5a plays a role in PMN recruitment. To determine whether C5a was sufficient to increase PMNs within the intestine, $C5^{-/-}$ mice subjected to IR were treated with C5a immediately prior to beginning reperfusion. Surprisingly, C5a treatment did not result in increased neutrophil infiltration after 2 h reperfusion (Fig. 3A). Therefore, despite the ability of C5a to induce local injury in response to IR, 1 μg C5a was not sufficient to recruit PMNs into the intestinal tissue.

Infiltration of PMNs into tissues results in increased oxygen radical production and local tissue damage. Increased oxygen radical production can be measured by determining the production of peroxidase (a subsequent product) by the tissue. As shown previously, IR induces increased tissue peroxidase generation and C5 is required for this increase (Fig. 3B and [11]). Similar to the ability of C5aRa to prevent IR-induced neutrophil infiltration, C5aRa inhibited tissue peroxidase generation. $C5^{-/-}$ mice subjected to laparotomy only (sham) had increased basal levels of tissue peroxidase that did not increase further following IR (Fig. 3B). Also, C5a, administered just prior to reperfusion, did not alter $C5^{-/-}$ mouse intestinal peroxidase production (Fig. 3B). Therefore, C5a is not sufficient to induce either PMN infiltration or increased peroxidase production during intestinal reperfusion.

C5a treatment causes local release of chemotactic factors but does not induce the expression of vascular adhesion molecules

Next we asked whether PMN infiltration did not occur in C5−/− mice treated with C5a because of an alteration in production of eicosanoids, LTB4 and PGE2. Data shown in Fig. 4A and B indicate that C5a alone induces the production of the chemoattractant, LTB4 and immunomodulator,

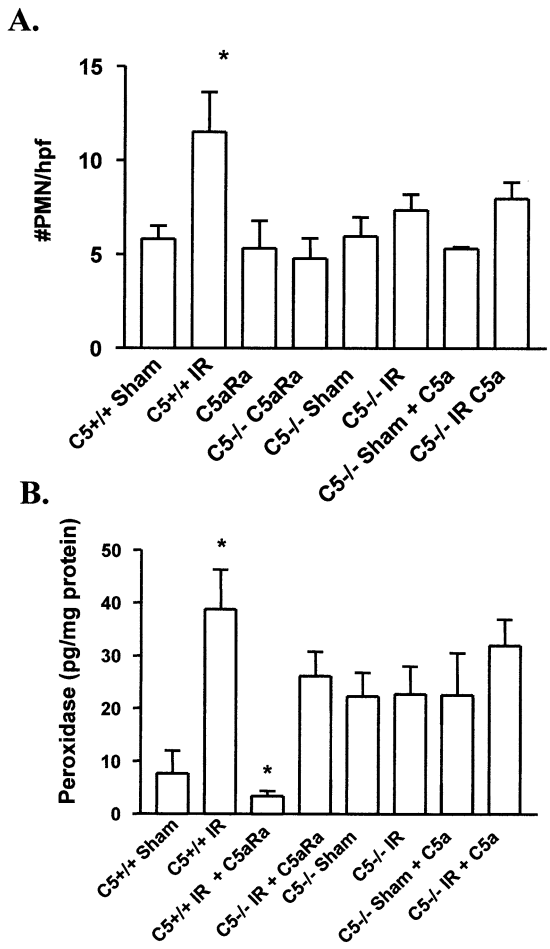

Fig. 3. Local C5a is required for PMN infiltration and increased tissue peroxidase production. (A) Intestinal sections from each treatment group were fixed in cold paraformaldyhyde for 2 h prior to sectioning and were staining with FITC-labeled GR-1 mAb. The number of PMN/high power field was counted in three fields per slide. (B) Peroxidase levels were determined as described in Material and Methods from in vitro incubation of intestinal sections obtained from mice after 2 h of reperfusion. Each bar is the average ± SEM of five to eight animals/group. Using ANOVA with Newman-Keuls post hoc test, * indicates significant difference from sham group, $P < 0.05$. ϕ indicates significant difference from IR treatment of $C5^{+/+}$ mice $P < 0.05$.

PGE2, indicating that their production alone is not sufficient to establish PMN infiltration. The eicosanoid production was not increased after IR. Administration of C5aRa decreased the production of LTB4 but not PGE2, suggesting that different C5a receptors [35] may be involved in the production of these eicosanoids. The administration of C5aRa to C5 deficient mice did not significantly alter production of either LTB4 or PGE2.

Besides chemotactic factors, the expression of endothelial adhesion molecules is required for tissue PMN infiltration. Because we noted that C5a induced a chemotactic eicosanoid (LTB4) without PMN infiltration, the expression of IR-induced adhesion factor, VCAM, was determined. As shown previously, C5 is required for VCAM expression 2 h after initiating reperfusion (Fig. 5A and B and [11]). C5aRa

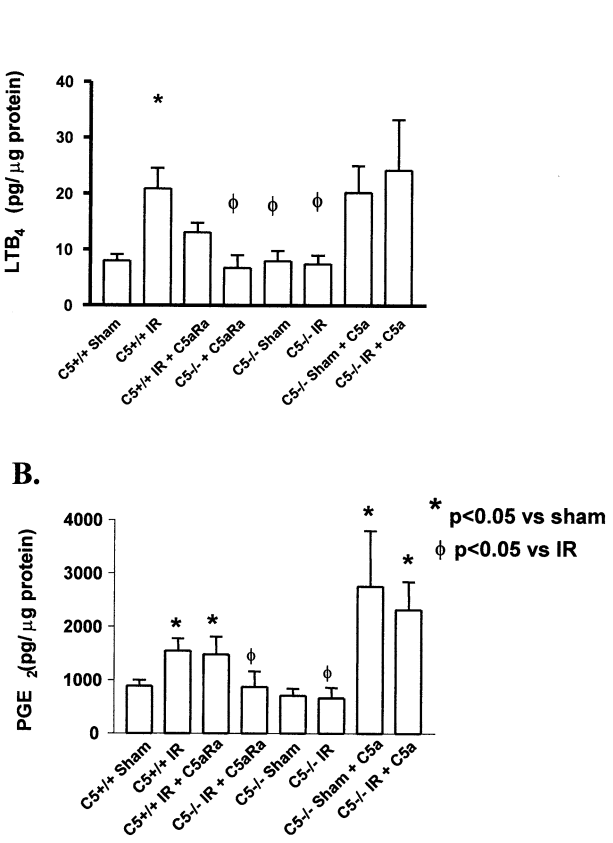

Fig. 4. C5a induces the production of chemotactic eicosanoids, LTB4 and PGE2. Eicosanoid, LTB4 (A) and PGE2 (B), production by tissue sections from each treatment group was determined by EIA as described in Materials and Methods. Each bar is average ± SEM with $n = 4-6$ animals/group. Using ANOVA with Neuman Keuls post hoc test, * indicates significant difference from sham treatment group, $P < 0.05$. ϕ indicates significant difference from IR treatment of $C5^{+/+}$ mice.

decreased VCAM expression within the intestine endothelium, indicating that C5a plays a role in this increased expression (Fig. 5C). However, C5a was not sufficient to induce VCAM expression on the intestinal endothelium (Fig. 5D–F). This set of experiments suggests that VCAM expression may be essential for the infiltration of PMNs into injured tissues.

C5a is not sufficient to induce lung permeability subsequent to IR.

A sequel of mesenteric IR is the remote organ injury that is readily recognized by assessing the permeability of the lung to macromolecules such as Evan's blue dye-bound albumin. Similar to previous results, mesenteric IR induced lung damage as indicated by an increase in the lavage/serum ratio of dye [11]. Previously, we showed that IR induces this damage in $C5^{+/+}$ mice but the damage is not present in $C5^{-/-}$ mice (Fig. 6 and [11]). Lung damage was decreased but not eliminated when $C5^{+/+}$ mice were treated with C5aRa, indicating that C5a has a role in the magnitude of damage. In contrast to the increased intestinal damage following IR, C5a administration to $C5^{-/-}$ mice prior to reperfusion was not sufficient to induce increased lung permeability (Fig. 6).

C5a induces VCAM expression on lung endothelium

We have shown previously that IR induces VCAM expression on lung endothelium at 2 h after beginning reperfusion [11]. Because C5a does not induce intestinal VCAM expression in response to IR, it was likely that C5a also did not induce VCAM on remote organ endothelium. We treated $C5^{-/-}$ mice with C5a immediately prior to the 2-h reperfusion period. As indicated previously, IR induces VCAM expression on lung endothelium of $C5^{+/+}$ but not $C5^{-/-}$ mice (Fig. 7 and [11]). In contrast to VCAM expression on the intestinal endothelium, administration of C5aRa blocked expression of this adhesion molecule in the lung (Fig. 7C). Treatment of $C5^{-/-}$ mice with C5a immediately prior to reperfusion induced VCAM expression (Fig. 7F). The up-regulation of VCAM expression was not seen in $C5^{-/-}$ mice treated with C5a and subjected to sham treatment (data not shown). Therefore, in conjunction with IR, C5a is sufficient to induce VCAM expression on lung endothelium but not in the intestine.

Peripheral blood neutrophil activation state

We have shown previously that IR increases PMN oxygen radical production and expression of adhesion molecules that may be involved in the development of remote organ injury [11]. Therefore, we examined the ability of C5a, produced in response to IR, to cause hydrogen peroxide production and expression of the integrin $\alpha 4$ adhesion molecule, CD49d by peripheral blood PMN. As indicated in Fig. 8, $C5^{+/+}$ mice had a significant increase in hydrogen peroxide generation in response to IR. This increase was inhibited by treatment with C5aRa, indicating that C5a plays a role in this induction. In contrast, $C5^{-/-}$ mice did not have an increased oxygen radical response to IR (Fig. 8). However, C5a treatment immediately prior to reperfusion of the $C5^{-/-}$ mice did not increase the production of oxygen radicals at 2 h after initiating reperfusion.

Similar to previous results, neutrophils from $C5^{+/+}$ mice expressed significantly more integrin $\alpha 4$ after IR [11]. As with hydrogen peroxide production, this increase was inhibited by C5aRa. Administration of C5a to $C5^{-/-}$ mice subjected to IR induced significant production of integrin $\alpha 4$ expression. There was no significant increase in integrin $\alpha 4$ adhesion molecule expression after C5a treatment of sham operated $C5^{-/-}$ mice. These experiments indicate that C5a administration to $C5^{-/-}$ mice subjected to IR does not cause remote lung injury, possibly due to the inability of peripheral blood PMN to produce hydrogen peroxide al-

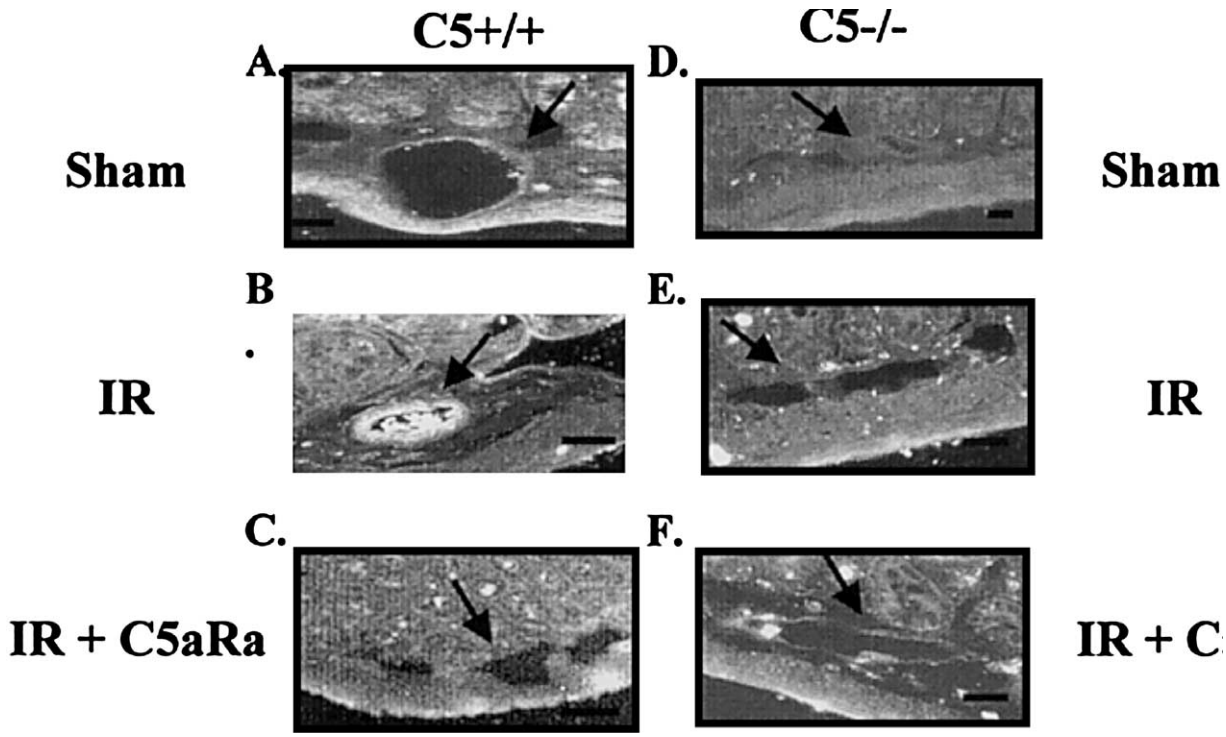

Fig. 5. IR-induced intestinal VCAM expression is not induced by C5a. Intestinal sections were stained for VCAM expression as described in Material and Methods. Stained sections from each group (A) $C5^{+/+}$ sham; (B) $C5^{+/+}$ IR; (C) $C5^{+/+}$ IR + C5aRa; (D) $C5^{-/-}$ sham; (E) $C5^{-/-}$ IR; (F) $C5^{-/-}$ IR + C5a are shown. Each photomicrograph is representative of three separate experiments. Original magnification is 400X.

though they upregulate the expression of adhesion molecules on the surface membrane. Therefore, despite a role for C5a in increased neutrophil activation, it is not sufficient to sustain full activation and subsequent remote organ injury.

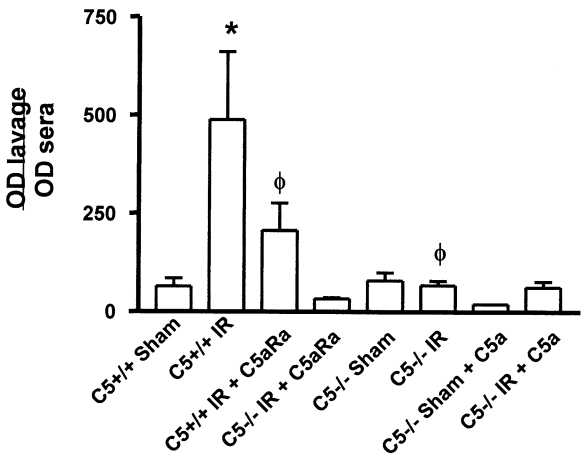

Fig. 6. C5a is necessary but not sufficient for increased macromolecular permeability after IR. Mice from each treatment group were injected iv with 0.1 ml Evans blue dye, 1 h prior to harvest. After one additional hour of reperfusion, the sera and bronchial lavage fluid were collected and the ratio of blue in each determined. Each bar is the average ± SEM with four to six animals/group. Using ANOVA with Neuman-Keuls post hoc test, * indicates significant difference from sham group. ϕ indicates significant difference from IR treatment of $C5^{+/+}$ mice.

Discussion

The role of C5a in the expression of tissue damage was assessed in two sets of experiments. In the first, C5aRa inhibited the action of C5a that is produced in response to mesenteric IR while the effect of C5b and the subsequently formed C5b-9 was not affected. In the second, the effect of C5a was determined in the absence of the formation of C5b-9 as it was administered to $C5^{-/-}$ mice. The fact that C5aRa is specific for mouse C5a receptor (CD88) albeit with low affinity [28,36] and the possibility that there may be more than one C5aR [35] on the surface membrane of intestinal cells and PMN make the results of the second set of experiments more powerful than the first.

The experiments reported here present three novel points. First, C5a has an active role in inducing damage that is independent of PMN infiltration and activation. Second, although C5a failed to increase tissue peroxidase production, C5a upregulated CD49d on peripheral blood PMN. Third, we show that C5a administered to animals undergoing IR induces VCAM expression on the lung endothelium but not on the intestinal vascular endothelium.

The role of C5a in tissue injury has been attributed to its ability to induce PMN infiltration and subsequent inflammation [15,18,23]. Our data indicate a role for C5a in mucosal damage that is distinct from its PMN chemotactic properties. Whereas treatment with C5aRa prevents PMN

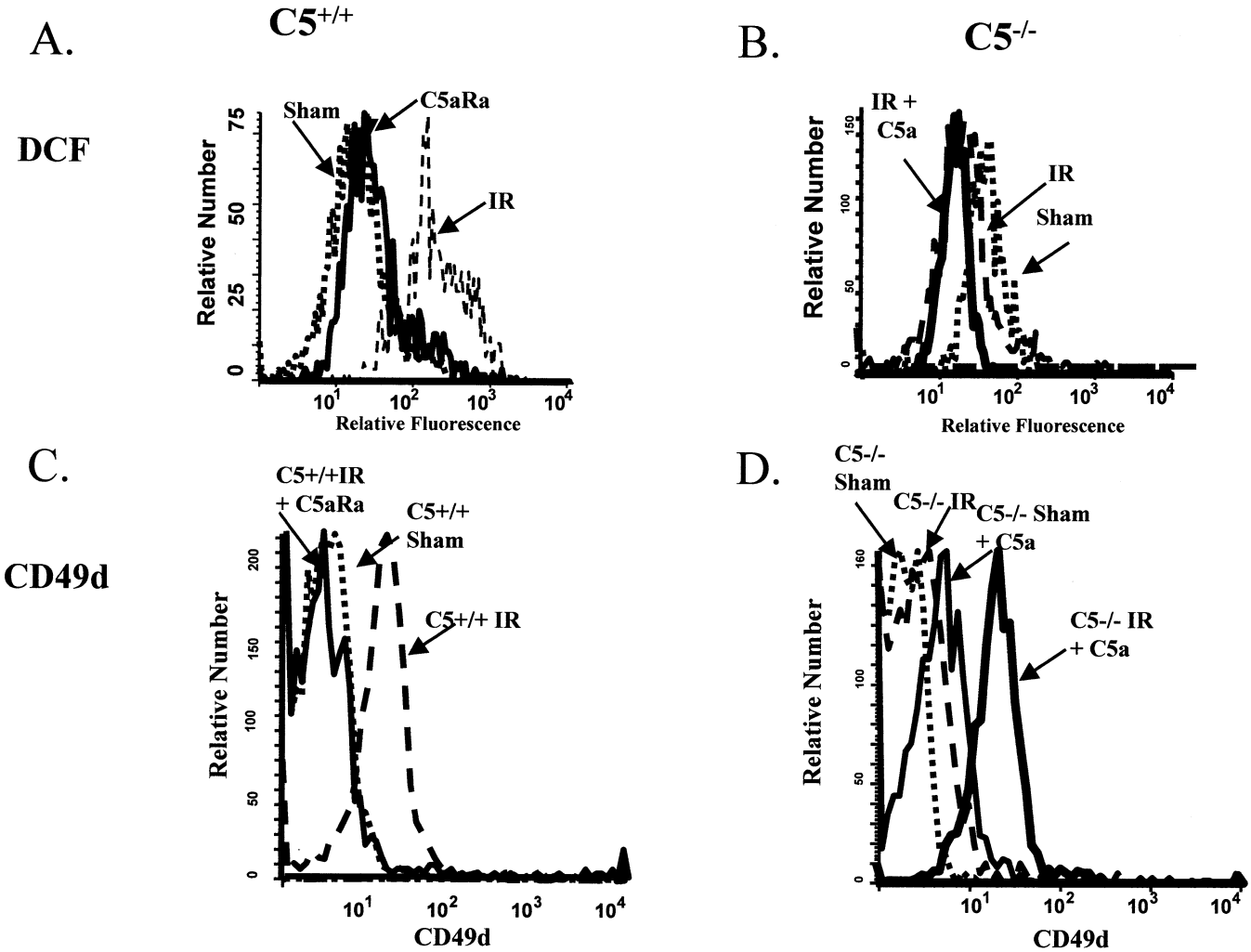

Fig. 7. C5a plays a role in IR-induced VCAM expression in the lung. Lung sections from each treatment group were stained for VCAM expression as described in Material and Methods. Stained sections from each group (A) $C5^{+/+}$ sham; (B) $C5^{+/+}$ IR; (C) $C5^{+/+}$ IR + C5aRa; (F) $C5^{-/-}$ sham; (E) $C5^{-/-}$ IR; (D) $C5^{-/-}$ IR + C5a are shown. Each photomicrograph is representative of three separate experiments. Original magnification is 400X.

infiltration, C5a reconstitution of $C5^{-/-}$ mice did not restore the IR-induced PMN infiltration or VCAM expression despite local production of the chemotactic factors, LTB4 and PGE2. There are probably multiple reasons for the discrepancy between the action of C5aRa in C5 sufficient mice and C5a reconstitution in C5 deficient mice undergoing IR. In our experiments, a single dose of C5a was given systemically and may not have reached sufficient local concentrations within the intestine. In addition, administration of C5aRa into C5 sufficient mice obviously allows the production of C5b and the subsequent formation of the membrane attack complex. The membrane attack complex can both destroy live cells and initiate biochemical processes that may damage the cells [37]. Although C5aRa did not have any effect when given to $C5^{-/-}$ mice, it is still possible that more than one C5aR exists on the surface of cells and the C5a that is produced during IR acts through another receptor. In addition, although it specifically inhibits the actions of C5a, it is possible that C5aRa prevents activation of other receptors. Either way, our data clearly show that local mucosal damage occurs despite the absence of a PMN infiltrate and in the absence of the membrane attack complex.

Similar to local injury, C5a is involved in peripheral PMN activation. C5aRa treatment of $C5^{+/+}$ mice decreased IR-induced upregulation of PMN oxygen radical production and CD49d adhesion molecule expression indicating that C5a has a role in peripheral blood PMN activation. C5a reconstitution of $C5^{-/-}$ mice also indicated that C5a is necessary and sufficient for the induction of increased CD49d expression on the cell surface of PMN. Our studies also indicate that C5a induces LTB4 expression. In vitro studies show that both C5a and LTB4 can also induce $\alpha 4$ integrin expression [38], suggesting that C5a has direct and indirect effects on peripheral PMN. The C5a-induced expression of additional adhesion molecules on circulating PMN has been shown previously in other models of inflammation [15,38].

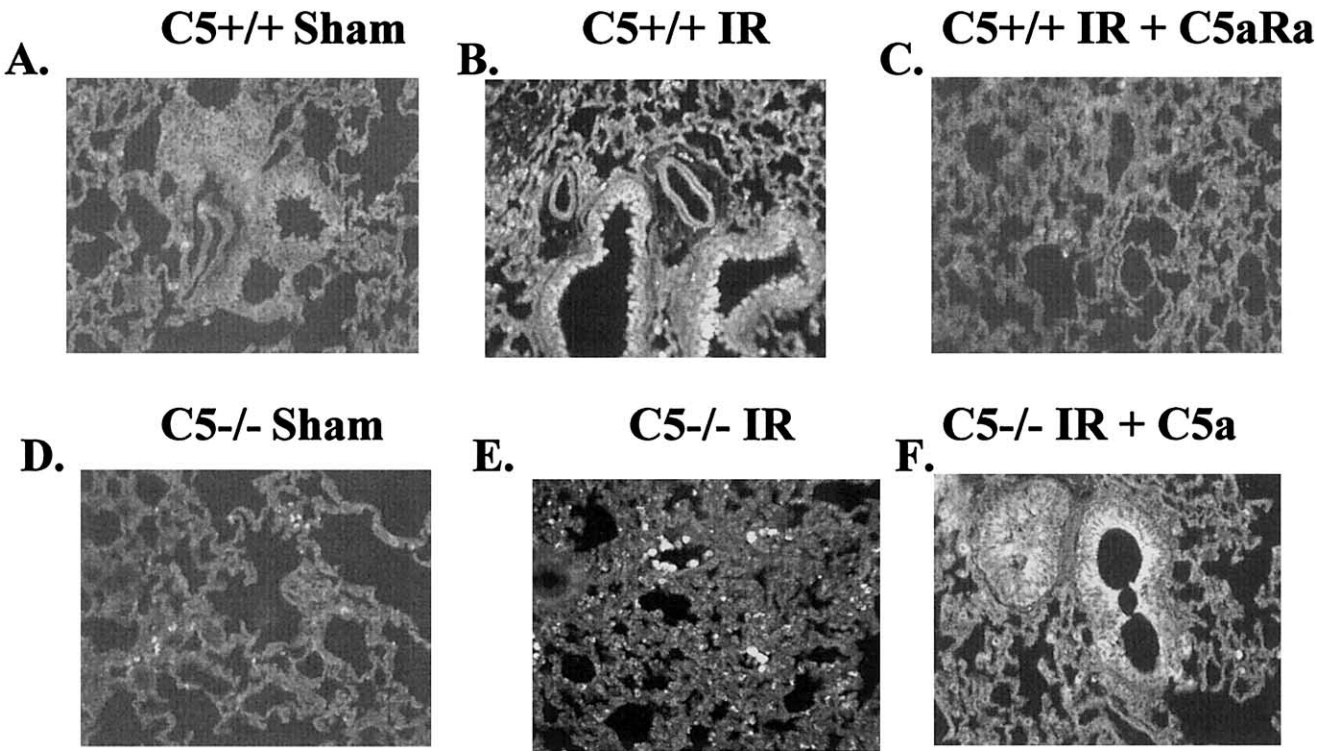

Fig. 8. IR-induced H_2O_2 production and CD49d expression are altered by C5a. Blood was obtained from $C5^{+/+}$ mice (A,C) and $C5^{-/-}$ mice (B,D) after sham treatment (---), IR (--) or IR + C5aRa or C5a (-) and treated as described in Material and Methods. The fluorescence of DCF as an indication of H_2O_2 production (A,B) and CD49d expression (C,D) was determined by flow cytometry. These data are representative of four to six experiments.

In contrast, there is a discrepancy between the two experimental approaches on the effect of C5a on PMN H_2O_2 production. Administration of C5aRa to C5+/+ mice decreased the oxygen radical production by PMN while C5a reconstitution of $C5^{-/-}$ mice was not sufficient to increase the oxygen radical production. A similar C5a-induced decrease in PMN release of H_2O_2 and neutrophil dysfunction has been found in a sepsis animal model [19]. As suggested by others, the dichotomy between the two models in H_2O_2 may be due to the systemic administration of C5a resulting in desensitization or dysfunction of the PMN [19]. An alternative possibility is that although a potent chemoattractant, C5a may also control PMN activation thus downregulating the immune response as indicated by the decreased H_2O_2 production. Taken together, these data indicate that CD49d upregulation is due to the presence of C5a and is independent of H_2O_2 production.

Unlike the local inflammatory response, C5a was sufficient for the IR-induced expression of VCAM on the lung endothelium. As shown previously, VCAM expression in response to IR requires the presence of C5 and as shown here, the inhibition of C5a with C5aRa in wild-type mice also inhibited this expression [11]. Furthermore, reconstituting C5−/− mice with C5a restored the IR-induced expression of VCAM. The fact that C5a administration caused VCAM expression in lung but not intestinal vascular endothelial cells indicates that either the concentration of C5a in the lungs is higher than that in the intestines or more likely, that VCAM expression requirements are different in the lung.

The finding of VCAM expression in the lungs as a result of intestinal IR and C5a is novel but not unexpected. The literature supports the induction of ICAM on the endothelium in response to IR and the induction of CD49d on PMN [9,11,15,17,32,39,40]. We have shown that both ICAM and VCAM are upregulated in response to IR, but during IR, only VCAM was dependent on C5a expression [11,32]. Although there is recent evidence that CD49d positive PMN do not require VCAM-1 for infiltration into tissues [41], the classic ligand for CD49d is VCAM-1. In recent studies of mesenteric or myocardial IR as well as other models of tissue injury, local VCAM-1 protein expression or its mRNA is increased during reperfusion [11,40,42,43]. In addition, a recent in vitro study showed that VCAM-1 mediates U937 cell binding to hypoxia/reoxygenation treated vascular endothelial cells [44]. The exact mechanism of VCAM-1 induction remains to be determined.

Thus, we conclude that C5a can induce local intestinal damage in response to IR that is independent of PMN. In addition, C5a induces expression of the adhesion molecule integrin α4 (CD49d) on peripheral PMN despite the absence of increased H_2O_2 production by the PMN. Finally, despite induction of CD49d on PMN and its ligand, VCAM on the lung epithelium, C5a is not sufficient to induce remote organ damage as assessed by vascular macromolecular leak.

Acknowledgments

The authors acknowledge the excellent technical assistance from Alicia Zamir, HM2 Osborne and Mr. Henry of the Histology Lab, Dept. of Pathology WRAIR/NMRC, and the Biomedical Instrumentation Center, USUHS.

References

[1] K.S. Kilgore, R.F. Todd, B.R. Lucchesi, Reperfusion injury, in: J.I. Gallin, R. Snyderman (Eds.), Inflammation: Basic Principles and Clinical Correlates, Lippincott Williams and Wilkins, Philadelphia, 1999, pp. 1047–1060.

[2] C. Kyriakides, W.G. Austen, Y. Wang, J. Favussa, F.D. Moore, H.B. Hechtman, Neutrophil mediated remote organ injury after lower torso ischemia and reperfusion is selectin and complement dependent, J. Trauma 48 (2000) 32.

[3] J.P. Williams, T.T.V. Pechet, M.R. Weiser, R. Reid, L. Kobzik, F.D. Moore, M.C. Carroll, H.B. Hechtman, Intestinal reperfusion injury is mediated by IgM and complement, J. Appl. Physiol. 86 (1999) 938–942.

[4] W.C. Conner, C.M. Gallagher, T.J. Miner, K.M. Tavaf-Motamen, H. Wolcott, T. Shea-Donohue, Neutrophil priming state predicts capillary leak after gut ischemia in rats, J. Surg. Res. 84 (1999) 24–30.

[5] W.L. Biffl, E.E. Moore, Role of the gut in multiple organ failure, in: A. Grenvik, S.M. Ayres, P.R. Holbrook, W.C. Shoemaker (Eds.), Textbook of Critical Care, 4th ed., W.B. Saunders Co., Philadelphia, 2000, pp. 1627–1635.

[6] C.S. Rinder, H.M. Rinder, B.R. Smith, J.C.K. Fitch, M.J. Smith, J.B. Tracey, L.A. Matis, S.P. Squinto, S.A. Rollins, Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation duringextracorporeal circulation, J. Clin. Invest. 96 (1995) 1564–1572.

[7] A.P. Vakeva, A. Agah, S.A. Rollins, L.A. Matis, L. Li, G.L. Stahl, Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy, Circulation 97 (1998) 2259–2267.

[8] A. Vakeva, S. Meri, Complement activation and regulator expression after anoxic injury of human endothelial cells, APMIS 106 (1998) 1149–1156.

[9] K. Wada, M.C. Montalto, G.L. Stahl, Inhibition of complement C5 reduces local and remote organ injury after intestinal ishcmenia/reperfusion in the rat, Gastroenterology 120 (2001) 126–133.

[10] Y. Wang, S.A. Rollins, J.A. Madri, L.A. Matis, Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease, Proc. Natl. Acad. Sci. 92 (1995) 8955–8959.

[11] S.D. Fleming, J. Anderson, F. Wilson, T. Shea-Donohue, G.C. Tsokos, C5 is required for CD49d expression on neutrophils and VCAM expression on vascular endothelial cells following mesenteric ischemia/reperfusion, Clin. Immunol. 106 (2002) 55–64.

[12] J. Kohl, Anaphylatoxins and infectious and non-infectious inflammatory diseases, Mol. Immunol. 38 (2001) 175–187.

[13] R.A. Wetsel, J. Kildsgaard, D.L. Haviland, Complement anaphylatoxins (C3a, C4a, C5a) and their receptors (C3aR, C5aR/CD88) as therapeutic targets in inflammation, in: J.D. Lambris, V.M. Holers (Eds.), Therapeutic Interventions in the Complement System, Humana Press, Totowa, NJ, 2000, pp. 113–153.

[14] R. Binder, A. Kress, G. Kan, K. Hermann, M. Kirschfink, Neurtophil priming by cytokines and vitamin D binding protein (Ge-globulin): impact on C5a-mediated chemotaxis, degranulation and respiratory burst, Mol. Immunol. 36 (1999) 885–892.

[15] S. Tyagi, L.B. Klickstein, A. Nicholson-Weller, C5a-stimulated human neutrophils use a subset of $\beta 2$ integrins to support the adhesion-dependent phase of superoxide production, J. Leukoc. Biol. 68 (2000) 679–686.

[16] M.C. Perianayagam, V.S. Balakrishnan, A.J. King, B.J.G. Pereira, B.L. Jaber, C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase-signaling pathway, Kidney Int. 61 (2002) 456–463.

[17] M. Tofukuji, G.L. Stahl, C. Metais, M. Tomita, A. Agah, c. Bianchi, M.P. Fink, F.W. Sellke, Mesenteric dysfunction after cardiopulmonary bypass: role of complement C5a, Ann Thorac. Surg. 69 (2000) 799–807.

[18] N.M. Bless, R.L. Warner, V.A. Padgaonkar, A.B. Lentsch, B.J. Czermak, H. Schmal, H.P. Friedl, P.A. Ward, Roles for C-X-C chemokines and C5a in lung injury after hindlimb ischemia-reperfusion, Am. J. Physiol. 276 (1999) L57–L63.

[19] M.S. Huber-Lang, J.V. Sarma, S.R. McGuire, K.T. Lu, R.F. Guo, V.A. Padgaonkar, E.M. Younkin, I.J. Laudes, N.C. Riedemann, J.G. Younger, P.A. Ward, Protective effects of anti-C5a peptide antibodies in experimental sepsis, FASEB J. 15 (2001) 568–570.

[20] M. Huber-Lang, J.V. Sarma, K.T. Lu, S.R. McGuire, V.A. Padgaonkar, R.F. Guo, E.M. Younkin, R.G. Kunkel, J. Ding, R. Erickson, J.T. Curnutte, P.A. Ward, Role of C5a in multiorgan failure during sepsis, J. Immunol. 166 (2001) 1193–1199.

[21] B.J. Czermak, V. Sarma, C.L. Pierson, R.L. Warner, M. Huber-Lang, N.M. Bless, H. Schmal, H.P. Friedl, P.A. Ward, Protective effects of C5a blockade in sepsis, Nat. Med. 5 (1999) 788–792.

[22] N.C. Riedemann, R.F. Guo, T.A. Neff, I.J. Laudes, K.A. Keller, J.V. Sarma, M.M. Markiewski, D. Mastellos, C.W. Strey, C.L. Pierson, J.D. Lambris, F.S. Zetoune, P.A. Ward, Increased C5a receptor expression in sepsis, J. Clin. Invest. 110 (2002) 101–108.

[23] M.S. Mulligan, E. Schmid, B. Beck-Schimmer, G.O. Till, H.P Friedl, R. Brauer, T.E. Hugli, M. Miyasaka, R.L. Warner, K.J. Johnson, P.A. Ward, Requirement and role of C5a in acute lung inflammatory injury in rats, J. Clin. Invest. 98 (1996) 503–512.

[24] E. Rothermel, O. Gotze, S. Zahn, G. Schlaf, Analysis of the tissue distribution of the rat C5a receptor and inhibition of C5a-mediated effects through the use of two MoAbs, Scand. J. Immunol. 52 (2000) 401–410.

[25] T.C. Pellas, W. Boyar, J.V. Oostrum, J. Wasvary, L.R. Fryer, G. Pastor, M. Sills, A. Braunwalder, D.R. Yarwood, R. Kramer, E. Kimble, J. Hadala, W. Haston, R. Moreira-Ludewig, S. Uziel-Fusi, P. Peters, K. Bill, L. Wennogle, Novel C5a receptor antagonists regulate neutrophil functions in vitro and in vivo, J. Immunol. 160 (1998) 5616–5621.

[26] T. Heller, M. Hennecke, U. Baumann, J.E. Gessner, A. Meyer zu Vilsendorf, M. Baensch, F. Boulay, A. Kola, A. Klos, W. Bautsch, J. Kohl, Selection of a C5a receptor antagonist from phage libraries attenuating the inflammatory response in immune complex disease and ischemia/reperfusion injury, J. Immunol. 163 (1999) 985–994.

[27] D.R. Haynes, D.G. Harkin, L.P. Bignold, M.J. Hutchens, S.M. Taylor, D.P. Fairlie, Inhibition of C5a-induced neutrophil chemotaxis and macrophage cytokine production in vitro by a new C5a receptor antagonist, Biochem. Pharmacol. 60 (2000) 729–733.

[28] T.V. Arumugam, I.A. Shiels, T.M. Woodruff, R.C. Reid, D.P. Fairlie, S.M. Taylor, Protective effect of a new C5a receptor antagonist against ischemia-reperfusion injury in the rat small intestine, J. Surg. Res. 103 (2002) 260–267.

[29] A.M. Finch, A.K. Wong, N.J. Paczkowski, S.K. Wadi, D.J. Craik, D.P. Fairlie, S.M. Taylor, Low-molecular-weight peptidic and cyclic antagonists of the receptor for the complement factor C5a, J. Med. Chem. 42 (1999) 1962–1974.

[30] D. Mastellos, J.C. Papadimitriou, S. Franchini, P.A. Tsonis, J.D. Lambris, A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration, J. Immunol. 166 (2001) 2479–2486.

[31] S. Rehrig, S.D. Fleming, J. Anderson, J.M. Gutheridge, J. Rakstang, C. McQueen, V.M. Holers, G.C. Tsokos, T. Shea-Donohue, Complement inhibitor, Crry-Ig attenuates intestinal damage after the onset of mesenteric ischemia/reperfusion injury in mice, J. Immunol. 167 (2001) 5921–5927.

[32] S.D. Fleming, T. Shea-Donohue, J.M. Gutheridge, L. Kulik, T.J. Waldschmidt, M.G. Gipson, G.C. Tsokos, V.M. Holers, Mice deficient in complement receptors 1 and 2 lack a tissue injury-inducing subset of the natural antibody repertoire, J. Immunol. 169 (2002) 2126–2133.

[33] C.-J. Chiu, A.H. McArdle, R. Brown, H.J. Scott, F.N. Gurd, Intestinal mucosal lesion in low-flow states, Arch. Surg. 101 (1970) 478–483.

[34] B.J. Czermak, A.B. Lentsch, N.M. Bless, H. Schmal, H.P. Friedl, P.A. Ward, Synergistic enhancement of chemokine generation and lung injury by C5a or the membrane attack complex of complement, Am. J. Physiol. 154 (1999) 1513–1524.

[35] S.A. Cain, P.N. Monk, The orphan receptor C5L2 has high affinity binding sites for complement fragments C5a and C5a des-Arg^{74}, J. Biol. Chem. 277 (2002) 7165–7169.

[36] T.M. Woodruff, A.J. Strachan, S.D. Sanderson, P.N. Monk, A.K. Wong, D.P. Fairlie, S.M. Taylor, Species dependence for binding of small molecule agonist and antagonists tot he C5a receptor on polymorphonuclear leukocytes, Inflammation 25 (2001) 171–177.

[37] F. Niculescu, H. Rus, Mechanisms of signal transduction activated by sublytic assembly of terminal complement complexes on nucleated cells, Immunol. Res. 24 (2001) 191–199.

[38] B. Johnston, P. Kubes, The α_4-integrin: an alternative pathway for neutrophil recruitment? Immunol. Today 20 (1999) 545–550.

[39] C.L. Ivey, F.M. Williams, P.D. Collins, P.J. Jose, T.J. Williams, Neutrophil chemoattractants generated in two phases during reperfusion of ischemic myocardium in the rabbit: evidence for a role for C5a and interleukin-8, J. Clin. Invest. 95 (1995) 2720–2728.

[40] R.A. Bowden, Z.-M. Ding, E.M. Donnachie, T.K. Petersen, L.H. Michael, C.M. Ballantyne, A.R. Burns, Role of α4 integrin and VCAM-1 in CD18-independent neutrophil migration across mouse cardiac endothelium, Circ. Res. 90 (2002) 562–569.

[41] B. Johnston, A. Chee, T.B. Issekutz, T. Ugarova, A. Fox-Robichaud, M.J. Hickey, P. Kubes, α_4 integrin-dependent leukocyte recruitment does not require VCAM-1 in a chronic model of inflammation, J. Immunol. 164 (2000) 3337–3344.

[42] C.D. Raeburn, C.M. Calkins, M.A. Zimmerman, Y. Song, L. Ao, A. Banerjee, X. Meng, A.H. Harken, Vascular cell adhesion molecule-1 expression is obligatory for endotoxin-induced myocardial neutrophil accumulation and contractile dysfunction, Surgery 130 (2001) 319–325.

[43] Z. Sun, X. Wang, A. Lasson, A. Bojesson, M. Annborn, R. Anderson, Effects of inhibition of PAF, ICAM-1 and PECAM-1 on gut barrier failure caused by intestinal ischemia and reperfusion, Scand, J. Gastroenterol. 1 (2001) 55–65.

[44] C.K.D. Ng, S.S. Deshpande, K. Irani, B.R. Alevriadou, Adhesion of flowing monocytes to hypoxia-reoxygenation-exposed endothelial cells: role of Rac1, ROS, and VCAM-1, Am. J. Physiol. 283 (2002) C93–C102.