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Membrane-Associated CD93 Regulates Leukocyte Migration and C1q-Hemolytic Activity during Murine Peritonitis

Mallary C. Greenlee-Wacker,* Carlos Briseño,∗ Manuel Galvan,∗+ Gabriela Moriel,∗ Peter Velázquez,∗+ and Suzanne S. Bohlson∗+

CD93 is emerging as a novel regulator of inflammation; however, its molecular function is unknown. CD93 exists as a membrane-associated glycoprotein on the surface of cells involved in the inflammatory cascade, including endothelial and myeloid cells. A soluble form (sCD93) is detectable in blood and is elevated with inflammation. In this study, we demonstrate heightened susceptibility to thioglycollate-induced peritonitis in CD93−/− mice. CD93−/− mice showed a 1.6–1.8-fold increase in leukocyte infiltration during thioglycollate-induced peritonitis between 3 and 24 h that returned to wild type levels by 96 h. Impaired vascular integrity in CD93−/− mice during peritonitis was demonstrated using fluorescence multiphoton intravital microscopy; however, no differences in cytokine or chemokine levels were detected with Luminex Multiplex or ELISA analysis. C1q-hemolytic activity in CD93−/− mice was decreased by 22% at time zero and by 46% 3 h after thioglycollate injection, suggesting a defect in the classical complement pathway. Leukocyte recruitment and C1q-hemolytic activity was restored to wild type levels when CD93 was expressed on either hematopoietic cells or nonhematopoietic cells in bone marrow chimeric mice. However, elevated levels of sCD93 in inflammatory fluid were observed only when CD93 was expressed on nonhematopoietic cells. Because cell-associated CD93 was sufficient to restore a normal inflammatory response, these data suggest that cell-associated CD93, and not sCD93, regulates leukocyte recruitment and complement activation during murine peritonitis. The Journal of Immunology, 2011, 187: 000–000.

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acute inflammation is a normal response to injury or infection characterized by recruitment of leukocytes to the site of injury, elimination of the pathogen or insult, and a return to normal tissue homeostasis. When improperly regulated, inflammation is involved in the progression of most diseases including cancer, heart disease, autoimmunity and sepsis (reviewed in Refs. 1–3). Therefore, deciphering the molecular mechanisms that regulate the inflammatory cascade has wide applicability to acute and chronic pathology. CD93, a type I transmembrane glycoprotein, is emerging as a novel regulator of inflammation. CD93 is expressed on a variety of cells involved in the inflammatory cascade, including neutrophils, monocytes, and endothelial cells. CD93, thrombomodulin (TM) and endosialin, constitute the group XIV family of C-type lectin-like domain (CTLD) containing proteins (reviewed in 4), characterized by a CTLD (referred to as D1), followed by a series of epidermal growth factor-like repeats (D2), a highly glycosylated mucin domain, a transmembrane region, and a short cytoplasmic tail. Several groups have demonstrated that TM is anti-inflammatory. For example, the CTLD of TM (TM-D1) inhibited LPS-induced proinflammatory signaling and complement activation (5–7), and cell-associated TM is a complement regulatory protein (8). Furthermore, mice deficient in TM-D1 (TM−/−;LeptD) are more susceptible to arthritis and sepsis (7). These observations led us to hypothesize that the other group XIV family members, such as CD93, share anti-inflammatory functions with TM.

In support of this hypothesis, recent studies demonstrated that the absence of CD93 led to increased inflammation and tissue destruction in a model of cerebral ischemia reperfusion injury; however, the mechanism responsible for CD93-dependent pathology was not defined (9). We previously demonstrated that CD93 is proteolytically cleaved from activated human monocytes and neutrophils (10), as well as mouse inflammatory macrophages. In addition, the soluble form of CD93 (sCD93) is elevated during inflammation (11). Recently, Jeon et al. (12) demonstrated that sCD93 is elevated in synovial fluid from rheumatoid arthritis patients and that human monocytes treated with sCD93 were more adhesive, phagocytic, and responsive to TLR ligands compared with control monocytes. These data support the hypothesis that CD93 regulates inflammation; however, its molecular function has remained elusive.

To investigate the molecular mechanism of CD93-dependent regulation of inflammation, we compared wild type (WT) and CD93−/− mice during thioglycollate-induced peritonitis. We observed increased leukocyte recruitment into the peritoneal cavity, dysregulated C1q-hemolytic activity, and altered vascular integrity in CD93−/− mice after induction of peritonitis compared with WT mice. Thioglycollate-induced peritonitis triggered a release of sCD93 only when CD93 was expressed on nonhematopoietic (radiation-resistant) cells. Moreover, expression of CD93 on either hematopoietic or nonhematopoietic cells was sufficient to restore both normal leukocyte infiltration into the peritoneal cavity and C1q-hemolytic activity. In this study we further delineate the molecular function of CD93 by demonstrating that CD93 is required to maintain vascular integrity and that the cell associated
molecule, and not the soluble protein, regulates leukocyte recruitment and complement activity.

Materials and Methods

Mice

CD93-deficient mice were provided by Dr. Marina Botto and Dr. Mark Walport (Imperial College, London, U.K.). CD93-deficient mice were backcrossed 11 generations on a Harlan C57BL/6 background. Genotype was confirmed by PCR using the following primers: P1 (5'-AGG GAT CCC AGC GAG GAA GGG CAA CTG-3') and P2 (5'-GAG GTC GCC AAC AAA AAG AC-3') for the WT band; and P1 and P3 (5'-GTC CTG GTA CCA GTC ATC TAT ATC-3') for the CD93-Δ7 band. Animals used in these studies were 8–12 wk old, except in bone marrow chimeras in which mice were 12–16 wk old. All studies were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee.

Reagents and Abs

All reagents were purchased from Fisher (Pittsburgh, PA) unless otherwise indicated. An Annexin V-FITC Apoptosis Detection Kit was purchased from BioVision (Mountain View, CA). Rat and sheep anti-mouse CD93 Abs were purchased from R&D Systems (Minneapolis, MN) and recombinant in PBS to 0.2 mg/ml. Rat anti-mouse CD11b-PE and IgG2b-PE Abs were purchased from Beckman (Fullerton, CA). Rat anti-CD93 (AA4.1)-PE, rat anti-mouse Gr1-PC5.5, rat anti-mouse IgG2b-PC5.5, rat anti-mouse F4/80-PE, and Rat IgG2a-PE Abs were purchased from eBioscience (San Diego, CA). HRP-conjugated donkey anti-sheep Ab was purchased from Jackson ImmunoResearch (West Grove, PA).

Generation and purification of recombinant proteins

Mouse CD93-D1.2 was amplified from the full-length cDNA using the primer set 5'-CTA GAA TAT ATT GAC GCC GCT TAC ACT TCA ACT GTG TGT-3' and 5'-ATC GGC GCC GCT TGC AAC AAG ACC CAT TGG G-3'. PCR products were digested using EcoRI and NotI and cloned into pcDNA 4 V5/His A expression vector (Invitrogen, Carlsbad, CA). Proteins were expressed in HEK293 cells after transfection with Lipofectamine (Invitrogen) and selection with 300 µg/ml Zeocin (Invitrogen). Stably transfected HEK293 cells were grown in 20% complete media and 80% serum-free media (293SFM II [Invitrogen] containing 10 mM HEPES, Pen/Strep, and 2 mM L-alanyl-L-glutamine [Mediatech, Manassas, VA]) for 48 h. His-tagged proteins were purified from culture supernatants by nickel chromatography using the ProBond purification system (Invitrogen) and fractions were assayed for CD93 by ELISA as described in (11). Positive fractions containing recombinant proteins were pooled and dialyzed versus PBS. Proteins were quantified with bicinchoninic acid (Pierce, Rockford, IL) against a standard curve of BSA, and purity was analyzed with SDS-PAGE and Western blot.

LPS-induced sepsis

WT mice were injected i.p. with 10 µg O111:B4 LPS per gram body weight (Sigma-Aldrich, St. Louis, MO). Septic mice were euthanized at 0, 6, and 18 h after injection of LPS, and sCD93 was measured by ELISA in peritoneal lavage fluid and serum.

Quantification of soluble proteins

Peritoneal lavage samples were tested using a Luminex Multiplex array according to the manufacturer’s protocol (Millipore, Billerica, MA) using a Luminex 200. CCL21 was quantified by ELISA according to the manufacturer’s instruction (R&D Systems, Minneapolis, MN). The mouse C1q ELISA was performed as described by Li et al. (14) with anti-C1q Abs: monoclonal rat anti-mouse C1q (Hyqut Biotechnology, Uden, The Netherlands) and 1151 (provided by Dr. Andrea Tenner, University of California, Irvine, CA). C3a and C5a levels were quantified using commercially available kits following the manufacturer’s protocol (Becton Dickinson, Franklin Lakes, NJ). The mouse sCD93 ELISA was performed as described by Greenlee et al. (11) with R&D sheep and rat anti-mouse CD93 Abs.

Vascular integrity intravital assay

At 6 h after i.p. injection of 1 ml sterile thiglycollate, animals were anesthetized with rodent mixture anesthesia (50 mg/kg ketamine, 10 mg/kg xylazine, 1.7mg/kg acepromazine) as described previously (15). The parietal peritoneum was surgically exposed by removing a lower abdominal skin flap, and it was visualized using an Olympus FV1000 inverted microscope equipped with a femtosecond-pulsed Ti:sapphire laser with dispersion compensation, an Olympus 25× 1.05NA water emersion XLPlan N objective, and four non-descanned detectors. The collagen surface of the parietal peritoneum was visualized via second-harmonic generation with 850 nm excitation and a 425–465 barrier filter. All subsequent images were excited at 800 nm. A low molecular weight, 10-kDa dextran-Texas red was injected retro-orbitally and visualized with a 575–625-nm barrier filter to highlight the vasculature and define imaging volume. Animals were then injected retro-orbitally with 150-µl AngioSense-680iVM (Perkin Elmer), and an image volume was acquired with a 675–700-nm barrier filter in 30-s intervals. At 2 min after AngioSense 680 iVM injection, images 50-µm beyond the collagen surface of the parietal peritoneum were quantified for relative fluorescence. Ten paired measurements inside and outside the blood vessel were analyzed using Imaris software (Bitplane) in each animal. Data are plotted as a ratio metric measurement to normalize for small differences in light scatter between specimens. The D’Agosto–Pearson omnibus test was used to determine whether the data points were distributed normally, and a two-sided Mann–Whitney U test with a 95% confidence interval was conducted to test for significance.

Hemolytic titer

Complement activity was measured with hemolytic titer. The C1q hemolytic titer was performed as described by Tenner et al. (16). Serial dilutions of mouse serum were prepared in GVB++ and then added to human C1q-depleted serum. Incubations with sheep erythrocytes (Colorado Serum Company, Denver, CO) opsonized with anti-sheep Abs (hemolysin, EA; Colorado Serum Company) were performed for 30 min at 37˚C. After centrifugation at 1800 × g for 3 min, supernatants were split in duplicate in 96-well plates, and the OD was measured at 412 nm. Percent of C1q lytic activity, corresponding to the percentage of EA lysis was calculated using the following formula: ([sample OD] – [buffer control OD]) / ([H2O lysis OD] – [buffer control OD]). C1q hemolytic activity, expressed as the z value of 1 (the dilution of serum at which 63% of EA were lysed), was calculated for all samples using a logarithmic regression analysis as

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described previously (16, 17). Results were expressed as a percent of C1q hemolytic activity relative to uninjected WT control serum. Serum samples were analyzed from a minimum of seven mice per time point. C3 hemolytic titers were performed using the same protocol with minimal modifications. Serial dilutions of mouse serum were prepared in GVB++ and then added to C3-depleted serum (C3D; Quidel, San Diego, CA). Incubation with EA was performed for 1 h at 37°C. Results of the C3 hemolytic titer were expressed as a percent of C3 hemolytic activity relative to uninjected WT mouse serum. Serum samples were analyzed from a minimum of seven animals per time point.

**Bone marrow chimera**

Bone marrow irradiation was performed on 4–8-wk-old recipient mice, and bone marrow was collected from donor mice at 4–6 wk of age. Mice were irradiated using the RAD SOURCE Technologies RS2000 irradiator. Mice were given two doses of radiation (600 cGy) at 115 cGy/min spaced 3 h apart. Mice were allowed to recover for 3 h after the final dose of irradiation and injected retro-orbitally with 0.2 ml bone marrow suspension (1 × 10^7 cells). Animals were maintained on low pH water (pH 2.5–3.0) for 3 wk to study the acute inflammatory response (18). To assess the contribution of CD93 deficiency results in increased leukocyte infiltration during peritonitis.

Leukocytes in peritoneal lavage fluid were counted at 24 and 96 h after i.p. injection of thioglycollate in CD93−/− mice (open bars) and WT mice (closed bars). Bars represent the mean leukocyte number for at least seven mice per genotype ± SEM over at least three separate experiments. ***p < 0.001.

**Statistical analysis**

Student t tests were used to calculate statistical significance unless otherwise indicated.

**Results**

CD93 deficiency results in increased leukocyte infiltration during peritonitis

I.p. injection of thioglycollate has been used widely as a model to study the acute inflammatory response (18). To assess the contribution of CD93 to the acute inflammatory response, WT and CD93−/− mice were subjected to thioglycollate-induced peritonitis. Leukocyte numbers in the peritoneal cavity were similar in WT and CD93−/− mice prior to injection of thioglycollate ([0.54 ± 0.11] × 10^7 versus [0.60 ± 0.13] × 10^7, respectively); however, increased leukocyte recruitment into the peritoneal cavity was observed at 3 and 6 h after injection of thioglycollate in CD93−/− mice compared with WT controls (1.5- and 1.8-fold, respectively; p < 0.0001; Fig. 1A). In a separate set of experiments, the number of leukocytes was counted in the peritoneal cavity at 24 and 96 h after injection of thioglycollate. The increase in leukocytes in the peritoneal cavity persisted 24 h after injection of thioglycollate in CD93−/− mice ([5.6 ± 0.35] × 10^7 versus [3.5 ± 0.23] × 10^7, respectively; p < 0.0001) and returned to WT levels after 96 h (Fig. 1B).

To further characterize this proinflammatory phenotype in the CD93−/− mice, cell populations in the peritoneal cavity were analyzed with microscopy. A normal inflammatory response was observed after thioglycollate challenge: there was an influx of neutrophils starting at 3 h after injection, followed by an influx of monocytes starting at 12 h after injection (Fig. 2A) (19). Although the total number of neutrophils and monocytes in the peritoneal cavity of CD93−/− mice was greater than WT (Fig. 2B, 2C), the relative percentage of each cell type was similar in WT and

**FIGURE 1.** CD93−/− mice show increased susceptibility to thioglycollate-induced peritonitis. A. Leukocytes in peritoneal lavage fluid from CD93−/− and WT mice were counted 0, 3, and 6 h after i.p. injection of thioglycollate (TG). Points represent the mean values from at least 12 individual mice per genotype over four separate experiments ± SEM. B, Leukocytes in peritoneal lavage fluid were counted at 24 and 96 h after i.p. injection of thioglycollate in CD93−/− mice (open bars) and WT mice (closed bars). Bars represent the mean leukocyte number for at least seven mice per genotype ± SEM over at least three separate experiments. ***p < 0.001.

**FIGURE 2.** Analysis of monocyte–macrophage and polymorphonuclear neutrophil (PMN) populations in the peritoneal cavity of CD93−/− and WT mice. Peritoneal cells were stained with Giemsa, and differential cell counts were performed. Two hundred cells per mouse were scored blinded by three separate individuals and the percentage of PMNs and monocytes (A) and total PMN and monocyte numbers (B, C) were plotted. Bars represent the mean of at least three mice per genotype ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
CD93<sup>−/−</sup> mice (Fig. 2A). Flow cytometric analysis of peritoneal cells yielded similar findings. At 6 and 24 h, the relative percentages of Gr1-positive neutrophils and F4/80-positive monocyte/macrophages, as well as annexin V-positive apoptotic cells, were equivalent between WT and CD93<sup>−/−</sup> mice (Supplemental Fig. 1). Because there was no difference in the relative percentage of neutrophils, monocytes, or apoptotic cells, the increase in leukocyte recruitment in CD93<sup>−/−</sup> mice is not due to augmented recruitment or persistence of a single cell type.

Cell-associated and sCD93 are elevated with inflammation

CD93 exists in two forms—a cell-associated full length form and a truncated soluble form. Cell-associated CD93 was measured by immunostaining for CD93 on tissue from the peritoneal cavity at 0 and 6 h after injection of thioglycollate. CD93<sup>+</sup> cells were detected at 6 h after injection of thioglycollate <sup>−/−</sup> WT mice. As expected, CD93 was absent from tissue in CD93<sup>−/−</sup> mice (Fig. 3). We previously demonstrated an increase in sCD93 concentration at 24 and 96 h after injection of thioglycollate (11). To further characterize the kinetics of sCD93 production, sCD93 was quantified at 0, 3, and 6 h after injection of thioglycollate using purified recombinant protein as a standard (Fig. 4A, 4B). sCD93 was low to undetectable at time zero and was elevated in peritoneal lavage fluid 3 and 6 h after thioglycollate challenge (20.9 ng/ml and 24.8 ng/ml [median value]; Fig. 4A). sCD93 was also elevated in peritoneal lavage fluid of WT mice 6 and 18 h after LPS-induced sepsis (6.5 ng/ml and 24.8 ng/ml [median], respectively; Fig. 4C). sCD93 was elevated in serum during LPS-induced sepsis.
GMCSF, KC, and MIP1-

lavage fluid of CD93

Fig. 2). Therefore, heightened susceptibility to thioglycollate-

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peritoneal lavage fluid (IL-1, IL-5, IL-6, IL-10, TNF-

array. The majority of cytokines and chemokines measured in

CD93 after injection of thioglycollate, whereas IL-4 was detectable only

two independent mouse models, and that sCD93 is preferentially

elevated at the site of inflammation compared with the concentra-

tion of sCD93 detected systemically during thioglycollate-

induced peritonitis.

Detection of cytokines and chemokines in the peritoneal fluid

Increased leukocyte chemotaxis is influenced by changes in the

cytokine and chemokine milieu; therefore cytokine (IL-1, IL-4, IL-

5, IL-6, IL-10, and TNF-α) and chemokine (MCP-1, RANTES,

GMCSF, KC, and MIP1-α) levels were measured in the peritoneal

lavage fluid of CD93−/− and WT mice using a Luminex Multiplex

array. The majority of cytokines and chemokines measured in

peritoneal lavage fluid (IL-1, IL-5, IL-6, IL-10, TNF-α, MCP-1,

RANTES, GMCSF, KC, and MIP1-α) were detectable at 3 and 6 h

after injection of thioglycollate, whereas IL-4 was detectable only

at 6 h after injection. However, there were no statistically

significant differences in the cytokine and chemokine levels between

CD93−/− and WT mice (p > 0.05; Table I). IL-12 (p<0.05), IL-13,

and IFN-γ were also measured, but the concentration in the fluid

was below the limit of detection. Elevated levels of the chemokine

CCL21 have been implicated in mediating leukocyte recruitment

in CD93−/− mice during cerebral ischemia and reperfusion injury

(9), where CCL21 was elevated in CD93−/− mice both prior to

injury and during injury. In this model, CCL21 levels were

permissive in peritoneal lavage fluid from CD93−/− and WT mice

under both baseline and inflammatory conditions (Supplemental

Fig. 2). Therefore, heightened susceptibility to thioglycollate-

induced peritonitis in CD93-deficient mice did not correlate with

an alteration in proinflammatory cytokines and chemokines mea-

sured.

CD93-deficient mice have altered vascular integrity

Because CD93 deficiency led to increased cell recruitment, but

did not affect cytokine or chemokine levels, we hypothesized that

particularities of the endothelium in the mice correspond to in-

creased inflammation. Using fluorescence multiphoton intravitral

microscopy, WT and CD93−/− mice were examined for changes in

vascular integrity 6 h after injection of thioglycollate (Fig. 5).

Using the indicator dye Angiosense 680 IVM, a ratiometric

analysis of the relative fluorescence inside and outside the blood

vessels of the parietal peritoneum during thioglycollate-induced

peritonitis revealed a significant increase in Angiosense 680 IVM

permeability in CD93−/− mice (p < 0.0001; Fig. 5C). Although

an increase in the permeability of Angiosense 680 IVM was ob-

served, total protein concentration was equivalent in the peritoneal

lavage fluid of CD93−/− mice and WT controls at this time point

(1.8 ± 0.28 mg/ml and 1.9 ± 0.91 mg/ml, respectively). Similarly,

no changes in total protein were observed at 3, 12, and 24 h after

injection (Table II). These results demonstrate that vascular in-

tegrity is altered in CD93−/− mice during sterile peritonitis.

CD93-deficient mice have dysregulated C1q hemolytic activity

A significant decrease in C1q hemolytic activity was observed at

time zero in CD93−/− mice compared with WT controls (22 ±

26%; p < 0.05; Fig. 6A). At 3 h after injection of thioglycollate,

the difference was more pronounced. C1q hemolytic activity was

46 ± 20% lower in serum from CD93−/− mice compared with

(3.1–5.6-fold greater after injection of LPS), but was not elevated

in the serum of thioglycollate injected mice. These data demon-

strate that sCD93 concentration is elevated with inflammation in

two independent mouse models, and that sCD93 is preferentially

elevated at the site of inflammation compared with the concentra-

tion of sCD93 detected systemically during thioglycollate-

induced peritonitis.

Table 1. CD93−/− mice have similar levels of cytokines compared with WT mice after induction of peritonitis

<table>
<thead>
<tr>
<th>Conditions</th>
<th>WT (3 h)</th>
<th>CD93−/− (3 h)</th>
<th>WT (6 h)</th>
<th>CD93−/− (6 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>49 ± 19</td>
<td>61 ± 16</td>
<td>37 ± 26</td>
<td>61 ± 27</td>
</tr>
<tr>
<td>IL-6</td>
<td>(5.1 ± 0.43) × 10^4</td>
<td>(4.6 ± 0.58) × 10^4</td>
<td>(2.1 ± 1.7) × 10^3</td>
<td>(1.7 ± 1.6) × 10^3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>79 ± 6.9</td>
<td>80 ± 35</td>
<td>2.8 ± 1.0</td>
<td>5.5 ± 3.3</td>
</tr>
<tr>
<td>MIP1</td>
<td>(6.5 ± 2.0) × 10^3</td>
<td>(6.4 ± 0.82) × 10^3</td>
<td>(8.3 ± 5.8) × 10^2</td>
<td>(4.6 ± 3.6) × 10^2</td>
</tr>
<tr>
<td>IL-4</td>
<td>nd</td>
<td>nd</td>
<td>55 ± 28</td>
<td>66 ± 99</td>
</tr>
<tr>
<td>IL-5</td>
<td>42 ± 10</td>
<td>33 ± 11</td>
<td>55 ± 22</td>
<td>40 ± 17</td>
</tr>
<tr>
<td>RANTES</td>
<td>6.9 ± 1.1</td>
<td>12 ± 6</td>
<td>14 ± 6</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>46 ± 12</td>
<td>39 ± 25</td>
<td>18 ± 9</td>
<td>22 ± 16</td>
</tr>
<tr>
<td>IL-10</td>
<td>(5.9 ± 1.4) × 10^2</td>
<td>(4.3 ± 1.0) × 10^2</td>
<td>(4.3 ± 1.8)</td>
<td>37 ± 12</td>
</tr>
<tr>
<td>KC</td>
<td>(7.3 ± 3.8) × 10^3</td>
<td>(10 ± 1.6) × 10^1</td>
<td>(1.0 ± 0.5) × 10^2</td>
<td>91 ± 75</td>
</tr>
<tr>
<td>MIP1α</td>
<td>(7.5 ± 1.6) × 10^2</td>
<td>(7.6 ± 2.5) × 10^2</td>
<td>(1.4 ± 0.3) × 10^2</td>
<td>(1.6 ± 0.7) × 10^2</td>
</tr>
</tbody>
</table>

Chemokines and cytokines in peritoneal lavage fluid from WT and CD93−/− mice were measured with Luminex at 3 and 6 h after injection of thioglycollate. Each value represents the mean from at least three individual mice per genotype (pg/ml) ± SD (p > 0.05).

KC, keratinocyte-derived chemokine; nd, not detected.

FIGURE 5. CD93−/− mice have altered vascular integrity during peritonitis. WT (A) and CD93−/− mice (B) were examined by intravitral microscopy for the permeability of Angiosense-680 IVM at 6 h after injection of thioglycollate. Scale bars, 80 μm. C. The relative fluorescence of tracer dye was quantified inside and outside blood vessels at 50 μm past the collagen surface of the parietal peritoneum. Data are plotted as the ratio of fluores-
cence outside to inside the blood vessel. The increased fluorescence in CD93−/− mice indicates changes in vascular integrity. n = 3 mice per genotype. ***p < 0.0001.
WT mice ($p < 0.01$; Fig. 6B). There was no defect in C1q production in CD93$^{-/-}$ mice, because equivalent C1q protein concentration was detected in serum from CD93$^{-/-}$ and WT mice at time zero (Fig. 6C). The defect in C1q hemolytic activity in CD93$^{-/-}$ mice did not reflect a general defect in complement activity because C3 hemolytic activity was equivalent between WT and CD93$^{-/-}$ mice (Fig. 6D). To determine whether dysregulated C1q hemolytic activity resulted in increased production of anaphylatoxins, as would be expected if the components of the classical pathway were more readily consumed in CD93$^{-/-}$ mice, C5a protein concentration was measured with ELISA. C5a was not elevated in serum (Fig. 6E) or peritoneal lavage fluid.

<table>
<thead>
<tr>
<th>Hours after Injection of Thioglycollate</th>
<th>Protein Concentration in WT Peritoneal Lavage Fluid (mg/ml)</th>
<th>Protein Concentration in CD93$^{-/-}$ Peritoneal Lavage Fluid (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5.5 ± 1.5</td>
<td>4.0 ± 2.7</td>
</tr>
<tr>
<td>6</td>
<td>1.8 ± 0.28</td>
<td>1.9 ± 0.91</td>
</tr>
<tr>
<td>12</td>
<td>1.5 ± 0.73</td>
<td>2.3 ± 0.36</td>
</tr>
<tr>
<td>24</td>
<td>2.9 ± 1.4</td>
<td>2.3 ± 0.36</td>
</tr>
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</table>

Peritoneal lavage fluid was collected and subjected to bicinchoninic acid to determine the total protein concentration. Each value represents the mean of at least three mice per genotype ± SD.

**FIGURE 6.** C1q hemolytic activity is dysregulated in CD93$^{-/-}$ mice. C1q hemolytic activity was measured in serum from WT (closed squares) and CD93$^{-/-}$ mice (open circles) by hemolytic titer at time zero ($n = 5$ experiments; A) and between 0 and 6 h after injection of thioglycollate ($n = 3$ experiments; B). The concentration of C1q in serum was measured at time zero by ELISA ($n = 6$ mice per genotype; C), C3 hemolytic activity in serum was measured by C3 hemolytic titer (D), and concentration of C5a in serum was measured with ELISA (E) in WT and CD93$^{-/-}$ mice. For hemolytic titers, each point represents the mean of duplicate wells from an individual mouse, and for ELISAs each point represents the mean of triplicate wells from an individual mouse. The dotted line represents the mean of each group. *$p < 0.05$, **$p < 0.01$.**
A bioactive form of CD93 (soluble or full-length), and the cellular source of bioactive CD93 (hematopoietic or nonhematopoietic cells). Reconstitution of CD93−/− mice with recombinant mouse sCD93 failed to restore leukocyte migration to WT levels (data not shown); therefore, bone marrow chimeras were generated to determine the role of hematopoietic and nonhematopoietic cells expressed CD93 during leukocyte recruitment.

Eight weeks after bone marrow reconstitution, CD93−/− mice that received WT donor bone marrow expressed CD93 on leukocytes in blood and on BMDMs (Fig. 7A, 7B). CD93 expression on blood leukocytes and BMDMs from WT mice that received CD93-deficient donor bone marrow was indistinguishable from CD93 expression from CD93−/− mice that received CD93−/− donor bone marrow (Fig. 7A, 7B). sCD93 concentration in peritoneal lavage fluid and serum from chimeric mice was dependent on CD93 expression on the nonhematopoietic cells.

Mice that expressed CD93 on nonhematopoietic cells (WT recipients) had elevated levels of sCD93 in the peritoneal lavage fluid 3 h after injection of thioglycollate (45.2 ± 10.1 ng/ml [WT donor and WT recipient] and 48.8 ± 11.5 ng/ml [WT donor and CD93−/− recipient]). CD93−/− mice that received WT bone marrow had low or undetectable sCD93 in peritoneal lavage fluid, which was not elevated with inflammation (Fig. 7C). Similar findings were observed in serum: sCD93 was present in serum when CD93 was expressed on nonhematopoietic cells, whereas low (or undetectable) levels of sCD93 were present in CD93−/− recipient mice receiving WT donor bone marrow (Fig. 7D).

Expression of CD93 on either hematopoietic cells or nonhematopoietic cells restored leukocyte recruitment to WT levels. CD93−/− mice reconstituted with WT bone marrow and WT mice reconstituted with CD93−/− bone marrow both recruited only 62% of the leukocytes found in the inflamed peritoneal cavity of CD93−/− controls (Fig. 8A). Expression of CD93 hematopoietic or nonhematopoietic cells alone also reconstituted C1q-hemolytic activity to WT levels. There was no significant difference in C1q-hemolytic activity in serum from mice that expressed CD93 on hematopoietically derived or nonhematopoietically derived cells compared with WT controls. Irradiation and reconstitution did not alter the phenotype in the CD93−/− mice as a significant decrease
in C1q hemolytic activity was detected in CD93−/− controls (p < 0.05; Fig. 8B). Increased leukocyte migration in CD93−/− mice directly correlated with a decrease in C1q hemolytic activity at 3 h after injection of thioglycollate, suggesting that alterations in complement activity regulate the recruitment phenotype (Fig. 8, Supplemental Fig. 4; p < 0.05, one-tailed Pearson correlation test).

**Discussion**

This study demonstrates that CD93 is required for the regulation of acute inflammation in thioglycollate-induced peritonitis. In mice deficient in CD93, more leukocytes were recruited into the peritoneal cavity and vascular integrity was altered, whereas total protein, cytokine, and chemokine levels remained the same. This study also identifies a novel relationship between CD93 and classical complement activity in vivo, because mice deficient in CD93 show dysregulated C1q hemolytic activity that is correlated with leukocyte infiltration. sCD93 is elevated with inflammation, and the source of sCD93 in vivo is nonhematopoietic cells; however, these data suggest that cell-associated CD93, and not the soluble form, regulates leukocyte recruitment and complement activity.

Using the same model of thioglycollate-induced peritonitis shown in this study, Norsworthy et al. (20) showed that CD93-deficient mice had a defect in the engulfment of apoptotic cells when exogenous apoptotic cells were introduced into the inflamed peritoneal cavity. Therefore, the increase in leukocytes in the CD93−/− peritoneal cavity could result from a failure to efficiently clear apoptotic cells; however, there was no difference in the relative percentage of annexin V-positive cells in the inflamed peritoneum of WT and CD93−/− mice (Supplemental Fig. 1), suggesting that failure to clear apoptotic cells was not responsible for the increase in leukocytes in the CD93−/− peritoneal cavity during peritonitis. Norsworthy et al. (20) also assessed leukocyte recruitment into the peritoneal cavity, but did not observe enhanced neutrophil recruitment four hours following thioglycollate challenge. Extended kinetics, batch variability of thioglycollate, and differences in genetic background of the mice could account for the discrepancies between these two studies.

Consistent with our observation that CD93−/− mice show increased cellular recruitment into the peritoneal cavity following thioglycollate challenge, Harhausen et al. (9) found that CD93 deficiency led to increased infiltration of CD11b+ cells after cerebral ischemia reperfusion injury. Harhausen et al. (9) attributed enhanced leukocyte migration to CCL21, which was upregulated in brain tissue of nonischemic CD93-deficient mice and further upregulated ischemic CD93-deficient mice compared with WT controls. Although no difference in CCL21 was detected in peritoneal lavage fluid from CD93−/− and WT mice under baseline and inflammatory conditions (Supplemental Fig. 2), CCL21 cannot be discounted as an important factor in driving increased leukocyte recruitment. For example, tissue-specific elevation in CCL21 or the ratio of CCL21 in peritoneal lavage fluid to serum may contribute to enhanced migration.

In addition to the production of chemokines and cytokines, the complement system plays an important role in propagating the inflammatory response, and activation of the complement system is a tightly regulated process. In the current study, thioglycollate-induced peritonitis led to C1q consumption as detected by a decrease in C1q-hemolytic activity in mouse serum. C1q hemolytic activity was further decreased in CD93−/− mouse serum compared with WT serum, suggesting that there is a defect in classical pathway activation in CD93−/− mice. Despite dysregulated C1q-hemolytic activity, increased C3a and C5a did not accompany increased leukocyte recruitment in CD93−/− mice. In addition, consumption of C3 hemolytic activity with inflammation was not detected. C3 is the most abundant complement component in serum; therefore, moderate changes in C3 consumption may not have been detected in this assay.

In addition to a defect in C1q hemolytic activity, altered vascular integrity was observed in CD93-deficient mice compared with WT controls 6 h after injection with thioglycollate. Integrity of the vasculature was measured using Angiosense 680 IVM indicator dye. This 250-kDa dye has been used previously to quantify vascular integrity in a model of collagen-induced arthritis (21). Specific leakage of Angiosense 680 IVM into the extracellular space indicates a change in vascular integrity, because smaller molecules (e.g., 40-kDa dextrans) are usually confined to the vasculature (22). Although these changes could explain dysregulation of cellular recruitment into the peritoneum, total protein, chemokine, and cytokine levels were comparable between WT and CD93−/− mice, indicating that CD93 has a specific role in regulating the integrity of the vasculature. It is possible that excessive complement activation in a CD93-deficient environment contributes to the loss of vascular integrity, although C3 deposition was not detected in the peritoneum of WT or CD93−/− mice (data not shown).

Several recent studies have highlighted the role of CD93 as a regulator of inflammation; however, the source of active CD93 is unknown. Jeon et al. (12) recently showed that sCD93 was elevated in synovial fluid from rheumatoid arthritis patients. Moreover, recombinant sCD93 induced the adhesion and phagocytic capacity of monocytes (11, 12). In this study, we identified radiation-resistant, nonhematopoietic cells as the source of sCD93 in vivo because sCD93 is produced and elevated only when CD93 is expressed on radiation-resistant cells in chimeric mice. These data suggest that the endothelium, the major nonhematopoietic site of CD93 expression, is the source of sCD93. Therefore, this protein may be a useful biomarker to assess endothelial cell damage during inflammation. In support of this hypothesis, Mälarstig et al. (23) recently demonstrated that sCD93 concentration in plasma was predictive of risk for myocardial infarction and associated coronary artery disease.

In addition, the data presented suggest that sCD93, produced from endothelium, is not required to regulate leukocyte recruitment in vivo, because leukocyte recruitment at 3 h after injection of thioglycollate was comparable to WT controls when sCD93 concentration were negligible (CD93−/− recipient mice with WT donor bone marrow; Figs. 7, 8). Moreover, these data demonstrate that the CD93 null mutation does not lead to a global developmental defect that leads to dysregulated inflammation, because the inflammatory phenotype was reversed with WT bone marrow. Similarly, reconstitution of membrane-associated CD93 restored C1q hemolytic activity to WT levels (Fig. 8B). Therefore, this study demonstrates that cell-associated CD93 regulates leukocyte recruitment that correlates with C1q hemolytic activity during thioglycollate-induced peritonitis. Future studies are aimed at determining the mechanism by which complement activity is regulated in the CD93-deficient mice.

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**Disclosures**

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References