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Murine CD93 (C1qRp) Contributes to the Removal of Apoptotic Cells In Vivo but Is Not Required for C1q-Mediated Enhancement of Phagocytosis[1]

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Human CD93 (known as C1qRp) has been shown to be a phagocytic receptor involved in the in vitro C1q-dependent enhancement of phagocytosis. However, binding of CD93 to C1q and its function remain controversial. In this study, we have generated CD93-deficient mice (*CD93*$^{-/-}$) to investigate its biological role(s). The *CD93*$^{-/-}$ mice were viable and showed no gross abnormalities in their development. Thioglycolate-elicited peritoneal macrophages deficient in CD93 showed a similar enhancement in complement- and FcγR-dependent uptake of RBC to the wild-type macrophages when plated on C1q-coated surfaces suggesting that the lack of this receptor had no effect on these C1q-mediated events. There was no impairment in either complement- or FcγR-dependent phagocytic assays in vivo. By contrast, the *CD93*$^{-/-}$ mice had a significant phagocytic defect in the clearance of apoptotic cells in vivo (human Jurkat T cells and murine thymocytes: $p = 0.0006$ and $p = 0.0079$, respectively) compared with strain-matched controls. However, in vitro, the CD93$^{-/-}$ macrophages showed similar engulfment of apoptotic cells to wild-type macrophages. Furthermore, no supporting evidence for a role of CD93 as an adhesion molecule was found using intravital microscopy or analyzing peritoneal cell recruitment in response to three different inflammatory stimuli (thioglycolate, zymosan A, and IL-1β). Thus, our findings indicate that murine CD93 is expressed on the peritoneal macrophage, especially on thioglycolate-elicited cells, but does not appear to play a key role in C1q-mediated enhancement of phagocytosis or in the intercellular adhesion events tested. However, our results suggest that it may contribute to the in vivo clearance of dying cells. ***The Journal of Immunology*****, 2004, 172: 3406–3414.**

The binding of C1q to immune complexes or directly to certain microorganisms initiates the activation of the classical pathway of the complement system. By binding to Abs complexed with Ag, C1q is a key protein that links the activities of the adaptive and innate immune systems. However, in addition to its role in activating the classical pathway of complement, C1q has been found capable of triggering a variety of cellular responses in vitro that may be mediated by specific receptor/binding molecules on the cell surface. Although a number of molecules capable of interacting with C1q have been described in the literature (reviewed in Refs. 1 and 2), to date the physiological role of these interactions remains unknown or controversial. Among the several putative C1q receptors, a 126-kDa transmembrane glycoprotein, known as C1qRp, has been of particular interest.

Molecular characterization of the human C1qRp revealed that this molecule is a heavily *O*-glycosylated type I transmembrane protein consisting of unique C-type lectin domains, a tandem array of epidermal growth factor-like domains, and a single transmembrane domain followed by a short cytoplasmic tail (3, 4). The characterization of the rat and mouse homologues, known also as AA4, demonstrated that this molecule displays strong homology (67–87% identity) across species (5–8) suggesting that this receptor may play an important biological role.

Human C1qRp was originally reported to be involved in the C1q-mediated enhancement of phagocytosis (3), an observation based on inhibition experiments using specific anti-C1qRp mAbs. The same Abs were shown to inhibit the enhancement of phagocytosis mediated by mannose-binding lectin and pulmonary surfactant protein indicating that these ligands may modulate this cellular function via the same cell surface receptor, the C1qRp. However, despite its designation, no evidence for a direct interaction between C1q or these other ligands and C1qRp was provided in the initial report. On the contrary in subsequent studies, C1qRp was shown not to interact with C1q under physiological conditions (9, 10) and a change in the nomenclature for this molecule was proposed. Steinberger et al. (10) using a retroviral-based cDNA expression system demonstrated that the human C1qRp molecule is identical to CD93, a molecule of unknown function that was assigned its cluster designation number (CD93) in 1995 at the Fifth International Workshop on Human Leukocyte Differentiation Ags (Boston, MA). Therefore, in light of this recent evidence, we will refer to the human or the mouse C1qRp as CD93 throughout this manuscript.

Initially, human CD93 was shown to be selectively expressed by cells of myeloid lineage, endothelial cells, platelets (11), and microglia (12). Subsequent studies demonstrated that in humans and in mice CD93 is not present on tissue macrophages and that the

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predominant site of expression is the vascular endothelium (13–15). In addition, mouse CD93 (known also as AA4) was found to be present on primitive hemopoietic stem cells giving rise to the hypothesis that this molecule was involved in angiogenesis, endothelial cell migration, or adhesion (13).

In this study, we aimed to explore the molecular properties and biological function(s) of CD93 by generating mice lacking CD93 and analyzing their phenotype. The CD93-deficient (*CD93*$^{-/-}$) mice were fully viable, and showed no gross spontaneous abnormalities in their vascular development or in their capacity to recruit inflammatory cells into the peritoneum. In addition, no defects in the C1q-mediated enhancement of complement- or FcγR-dependent phagocytosis were found. Nevertheless, the CD93-deficient mice had an impaired uptake of apoptotic cells in vivo indicating that this receptor may contribute to the removal of dying cells.

Materials and Methods

Targeting construct and generation of CD93$^{-/-}$ *mice*

Mice with a disrupted *CD93* locus were generated by homologous recombination in embryonic stem (ES)[3] cells. The targeting vector was constructed using isogenic DNA isolated from a 129/Sv genomic DNA λ library (Stratagene, La Jolla CA). A 1128-bp fragment was deleted from exon 1 and replaced with a positively selectable marker, the neomycin-resistance gene cassette (pMC1NeoPolyA; Stratagene), flanked by 2.1- and 2.4-kb regions of target homology (see Fig. 1*A*). External to the 5′ region of homology was placed a negatively selectable gene, the HSV thymidine kinase gene. ES cells were transfected by electroporation with the linearized targeting vector. Colonies surviving positive/negative selections were screened by Southern blot of *Sca*I digests using a 5′ external probe. Recombinant ES clones were used for microinjection into 3.5-day postcoitus C57BL/6 blastocysts and chimeric mice were generated. Germline transmission was obtained and interbreeding of heterozygotes yielded viable mice homozygous for the disrupted allele. Subsequent genotyping was performed by PCR using the following primers: mC1qRp/W^{+} (5′-AGGG ATCCCAGCGAGGAAGGGCAACTG-3′), neomycin 3′ (5′-GGGATCG GCAATAAAAAGAC-3′), and mC1qRp/U^{-} (5′-GTCCTGGCACTCAT CTATATC-3′). Amplification of the wild-type gene resulted in an amplicon of ~721 bp while the targeted gene gave an ~480-bp fragment.

Mice

The CD93-deficient mice used were either on the (129/Sv × C57BL/6) hybrid genetic background, or on the pure inbred 129/Sv background, or backcrossed onto C57BL/6 for five generations as specified in each experiment. Age-, strain-, and sex-matched mice were used as controls in each experiment. Animals were maintained in specific pathogen-free conditions. All animal procedures were in accordance with institutional guidelines.

Abs and flow cytometric analysis

The Abs used in this study were as follows: 493 mAb (anti-CD93) biotinylated (16), generously donated by Prof. A. Rolink (Pharmazentrum, Basel, Switzerland), rat anti-mouse F4/80 FITC and rat anti-mouse 7/4 (both from Caltag Laboratories, TCS Biosciences, Buckingham U.K.). Rat anti-mouse CD11b-PE, a rat anti-mouse Thy1.2 biotinylated (53-2.1), rat anti-mouse CD45R (B220-PE), rat anti-mouse-CD4, rat anti-mouse CD8α, mouse anti-human CD3 biotinylated and rat IgM anti-CD24 (J11d), rat anti-mouse CD43 (S7), rat anti-mouse CD11c, rat anti-mouse DX-5 (NK cells), and rat anti-mouse CD14 (all from BD Biosciences/PharMingen, San Diego, CA). Biotinylated mAb were detected by streptavidin-CyChrome or streptavidin-allophycocyanin (BD Biosciences/PharMingen). The 2.4G2 rat anti-mouse CD16/32 (FcγII and III), 7G6 rat anti-mouse CD35/CD21 (CR1/CR2), and the two Abs used in the FcγR-dependent clearance experiments (mouse IgG2a anti-mouse erythrocytes (34-3C) for the in vitro experiments (17) and mouse IgG1 anti-mouse erythrocytes (34-3C) (18) for the in vivo studies) were all kindly donated by Prof. S. Izui (Department of Pathology, Centre Medical Universitaire, University of Geneva, Geneva, Switzerland). FACS analysis was performed using a FACSCalibur (BD Biosciences, San Jose, CA) and the data were analyzed using WinMDi software (version 2.8; The Scripps Research Institute, La Jolla, CA).

[3] Abbreviations used in this paper: ES, embryonic stem cell; MFI, mean fluorescence intensity; PMN, polymorphonuclear neutrophil.

Peritoneal cell recruitment experiments

One milliliter of sterile 4% Brewer's thioglycolate solution or 1 mg of zymosan A (Sigma-Aldrich, Poole, U.K.) or 10 ng of recombinant mouse IL-1β (R&D Systems, Abingdon, U.K.) was injected into the peritoneum of CD93-deficient and strain-matched control mice. Peritoneal lavages were performed at different time points up to 4 days postinjection by flushing the peritoneum with cold HBSS containing 5 mM EDTA. The total number of cells recruited was calculated by light microscopy on a hemocytometer. The different cell populations recruited were assessed on coded cytospin preparations stained with Diff-Quick (Dade-Behring, Marburg, Germany) and/or by flow cytometry.

Intravital microscopy

Leukocyte-endothelial cell interactions induced by intrascrotal administration of IL-1β (30 ng) were studied by intravital microscopy, as previously described (19). Postcapillary venules, 20–40 μm in diameter were analyzed. Briefly, rolling leukocytes were defined as those moving more slowly than the blood flow, while firmly adherent cells were those remaining stationary for 30 s or longer within a given 100-μm segment of a venule. Extravasated cells were defined as the leukocytes in the extravascular tissue within 50 μm of the 100-μm vessel segment under observation. Several different vessel segments from more than one vessel (range 3–5) were analyzed for each animal.

Phagocytosis assays

The phagocytic ability of thioglycolate-elicited peritoneal macrophages was investigated using the following in vivo and in vitro assays.

In vivo clearance of apoptotic cells. Human Jurkat T cells and mouse thymocytes were made apoptotic as previously described (20). The in vivo studies were conducted using the peritonitis model previously established in our laboratory (20). Briefly, sterile peritonitis was induced in the gene-targeted and strain-matched control animals by an i.p. injection of 1 ml of 4% thioglycolate. After 4 days the mice were injected i.p. with 10^7 apoptotic Jurkat T cells or 3×10^7 apoptotic murine thymocytes in a volume of 200 μl. In all experiments, unless otherwise stated, the mice were sacrificed after 20 min. The peritoneal cells were then recovered by lavage with ice-cold HBSS/5 mM EDTA. Phagocytosis was scored on coded cytospin preparations as previously described (20). The phagocytic intake was also estimated by FACS by fluorescent labeling the apoptotic cells before injection with CFSE (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's protocol (5 μM at 10^7 cells/ml) and by detecting macrophages with a PE-conjugated anti-CD11b (Mac1) Ab (BD Biosciences/PharMingen). To distinguish the apoptotic cells ingested by macrophages from those externally bound, the apoptotic cells on the surface of the macrophages were detected using a biotinylated anti-human CD3 (for Jurkat T cells) or a biotinylated anti-mouse Thy 1.2 (for murine thymocytes) Ab followed by an incubation with a streptavidin-CyChrome conjugate (BD Biosciences/PharMingen). To calculate the number of macrophages containing apoptotic cells, the double-positive apoptotic cells were then subtracted from the single-positive cells.

In vitro phagocytosis of apoptotic cells. Thioglycolated-elicited peritoneal macrophages were cultured overnight on coverslips in 24-well plates in RPMI 1640 10% FCS supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin at the concentration of 0.5×10^6cells/well. Apoptotic cells (1×10^6) (Jurkat T cells or murine thymocytes) in RPMI 1640 supplemented with 10% mouse wild-type or C1q-deficient serum were added per well and the plates were spun at $50 \times g$ for 3 min. After a 20-min incubation at 37°C, 5% CO_2, the plates were washed with cold PBS and the coverslips were mounted on microscope slides and stained with Diff-Quick. The percentage of apoptotic cells phagocytosed by macrophages was then assessed on coded cytospins as described previously (20).

In vitro FcγR- or C3-dependent phagocytosis on C1q-coated plates. Previously published assays were modified as follows (21). LabtekII eight-well chamber slides were coated for 2 h at room temperature with 40 μg/ml human C1q (Sigma-Aldrich) and then washed twice with PBS. Three hundred microliters of a 7.5×10^5cells/ml suspension obtained by peritoneal lavage of 4-day thioglycolate-elicited mice were added to each well. The slides were then spun at $50 \times g$ for 3 min and incubated at 37°C in 5% CO_2 for 1 h. One hundred twenty microliters of CFSE-labeled guinea pig RBC coated with C3 or 120 μl of CFSE-labeled murine RBC coated with a mouse IgG2a (34-3C), prepared as explained below, were added to the wells, spun at $50 \times g$ for 3 min and incubated for 30 min at 37°C 5% CO_2. The cells were recovered by trypsination and repeated washing with PBS/1% BSA. The noningested RBC were lysed with a hypotonic solution. The amount of RBC phagocytosed was then quantified by FACS analysis.

Macrophages were identified using a PE-conjugated anti-CD11b (Mac-1) Ab (BD Biosciences) and the phagocytic cells containing CFSE-labeled RBCs were seen as double positive. In selected experiments, the phagocytosis was also assessed by light microscopy on coded cytospins. On cytospin preparation, the number of RBCs ingested per 100 macrophages was defined as the phagocytic index, whereas the percentage of effector cells containing at least one erythrocyte was defined as the percent of phagocytosis. At least 300 macrophages were scored per well and duplicate wells for each experimental condition were used. The murine or guinea pig RBCs for the above experiments were prepared as follows. The RBCs were initially washed three times in PBS and then resuspended to 2% v/v. These cell suspensions were incubated with CFSE according to the manufacturer's protocol for 5 min at room temperature and then washed twice with PBS/1% BSA and resuspended to 0.1% v/v. For the complement-dependent experiments, 10 μl of C5-deficient mouse serum from DBA/2 mice were added to 50 μl of 0.1% (v/v) suspension of guinea pig red cells and incubated with gentle rotation at 37°C for 30 min. The level of C3 opsonization was checked by FACS using a biotinylated polyclonal anti-mouse C3 (Caltag Laboratories, TCS Biosciences) and hemoagglutination assays. For the FcγR-dependent uptake experiments, 1 ml of murine RBC suspension was incubated in PBS/1% BSA for 1 h at 4°C with 10 μg, 1 μg, or 100 ng of the 34-3C Ab (a murine IgG2a anti-mouse RBC) (18) which resulted in a high, medium, or low level of opsonization, respectively, as assessed by FACS using a biotinylated anti-mouse κ-chain-specific Ab (BD Biosciences). Opsonized RBC suspensions were then washed with PBS/1% BSA, resuspended to 0.1% v/v in RPMI 1640 containing 2 mM glutamine and 50 μg/ml gentamicin and used immediately in the phagocytic assays.

FcγR- or complement-dependent phagocytosis of murine RBCs in vivo. As for the in vitro experiments, murine RBCs were labeled with CFSE as described above and resuspended to 2% v/v in PBS/1% BSA. For the FcγR-dependent clearance, the cell suspension was then incubated for 1 h at 4°C with end-over-end rotation with 15 μg/ml mouse 34-3C IgG1 anti-murine red cells, which resulted in the lowest level of IgG opsonization required for the uptake in vivo. For the complement-dependent experiments, the RBCs, prepared in the same way, were instead opsonized for 30 min at 4°C with end-over-end rotation with 20 ng/ml of a rat IgM anti-murine CD24 (J11d), a subagglutinating amount of IgM that was capable of activating the complement system via the classical pathway in the peritoneal cavity. Following the incubation with the respective Abs, the cell suspensions were washed twice with PBS, resuspended to 2% v/v and 250 μl were injected into the peritoneum of each mouse. After 30 min, the peritoneal cells were recovered by flushing the peritoneum with ice-cold HBSS/5 mM EDTA. Noningested red cells were lysed with a hypotonic solution and the phagocytic uptake was measured by FACS as described previously for the in vitro FcγR- and complement-dependent experiments or by light microscopy on coded cytospins. In each experiment, C1q-deficient mice (22) and Fcγ chain-deficient mice, kindly provided by Dr. S. Verbeek (Department of Human and Clinical Genetics, Leiden University, Medical Center, Leiden, The Netherlands) (23), were used as negative controls for the complement- and the FcγR-dependent phagocytosis experiments, respectively. Conversely, the C1q-deficient mice and Fcγ chain-deficient mice were used as positive controls for the FcγR- and the complement-dependent phagocytosis experiments, respectively.

Cell binding assay

Apoptotic cell binding assays were performed following the same protocol used for the in vitro phagocytic assays except that the macrophages were maintained at 4°C to prevent the local release of opsonins, including complement (24). In brief, thioglycolated-elicited peritoneal macrophages were plated overnight at 0.5×10^6 cells/well on coverslips in 24-well plates. The following day, the cells were cooled to 4°C and washed three times with prechilled PBS and kept in RPMI 1640 without FCS at 4°C. Apoptotic thymocytes, prepared as described above, were resuspended in prechilled RPMI 1640 without FCS and added to the cells at a variable ratio from 1:1 up to 20:1 (apoptotic cells per macrophage) for 20 min on ice. After a 20-min incubation, unbound apoptotic cells were removed by extensive washing with cold PBS and the coverslips were mounted on microscope slides and stained with Diff-Quick. The percentage of apoptotic cells bound to macrophages was then assessed on coded cytospins.

Statistics

The data are presented as means ± SEM unless otherwise stated. All statistics were calculated using GraphPad Prism (version 2.0; GraphPad, San Diego, CA). Nonparametric tests were applied throughout with differences being considered significant for p values <0.05.

Results

Gene-targeted CD93-deficient mice develop normally and have no gross abnormalities in their cellular distribution

The murine homologue of the human *CD93* gene was disrupted in 129/Sv ES cells by homologous recombination with the targeting vector described in Fig. 1*A*. This vector was designed to cause a 1128-bp deletion in exon 1 of the mouse *CD93* gene following homologous recombination. Recombinant ES cell clones were identified using Southern blotting of informative *Sca*I-digested DNA probed with a 5′ external genomic probe. In the wild-type allele, this probe detected a *Sca*I-fragment of 6 kb whereas in the recombinant allele, due to the loss of a *Sca*I site within the deleted portion of exon 1, a larger 11-kb fragment was seen (data not shown). Following microinjection of the recombinant clones into C57BL/6 blastocysts, chimeric mice were generated and germline transmission of the disrupted allele achieved. Homozygous CD93-deficient mice were viable and fertile under specific pathogen-free conditions. The disrupted allele was inherited in accordance with normal Mendelian inheritance patterns. The absence of CD93 was confirmed at the molecular level by RT-PCR analysis in thioglycolate-elicited peritoneal macrophages (data not shown) and at the protein level by FACS analysis of bone marrow-derived B220-positive immature B cells stained with 493, a mAb anti-mouse CD93 (14, 16) (Fig. 1*B*). Because CD93 is expressed on primitive multipotential hemopoietic precursors capable of giving rise to the entire spectrum of mature cells of the blood system (13), a comparative analysis of the cellular distribution in various compartments between *CD93*$^{-/-}$ and the control mice was conducted by flow cytometry. A summary of the data is shown in Table I. Although this analysis did not examine each compartment in depth, no gross abnormalities were detected in the maturation and distribution of bone marrow-derived cells.

Mouse CD93 expression on activated peritoneal macrophages

Human CD93 was originally described as a receptor for C1q that was involved in mediating enhanced phagocytosis by peripheral blood monocytes/macrophages (4, 25). However, recent reports showed that in humans, rats, and mice, CD93 is not expressed by tissue macrophages (7, 9, 14, 15). In view of these controversial findings, we started our investigations by assessing by FACS CD93 expression on resident and thioglycolate-elicited peritoneal macrophages using the 493 mAb and the *CD93*$^{-/-}$ animals as negative controls. This analysis confirmed that CD93 was expressed on resident peritoneal macrophages (Fig. 2*A*), although at a very low level (wild-type mean fluorescence intensity (MFI): 34.62 ± 1.59; CD93$^{-/-}$ MFI: 29.24 ± 1.250, $p = 0.01$), and that its expression was influenced by the activation state of the macrophages (MFI of CD93 expression on thioglycolate-elicited wild-type macrophages was 156.0 ± 4.46 compared with 24.66 ± 2.17 on CD93-deficient macrophages used as negative controls, $p < 0.0001$) (Fig. 2*B*).

In vitro C1q-dependent enhanced phagocytosis of IgG- or C3-opsonized RBC

Having established that peritoneal thioglycolate-elicited macrophages express CD93, we used these cells for all subsequent experiments to investigate the proposed phagocytic role of this molecule. Initially, we examined whether the CD93 was indeed the receptor mediating the C1q-dependent enhancement of phagocytosis as previously demonstrated by blocking the receptor with specific Abs. We used a phagocytic assay similar to that described by Guan et al. (25). Thioglycolate-elicited macrophages obtained from *CD93*$^{-/-}$ mice and strain-matched controls were incubated for

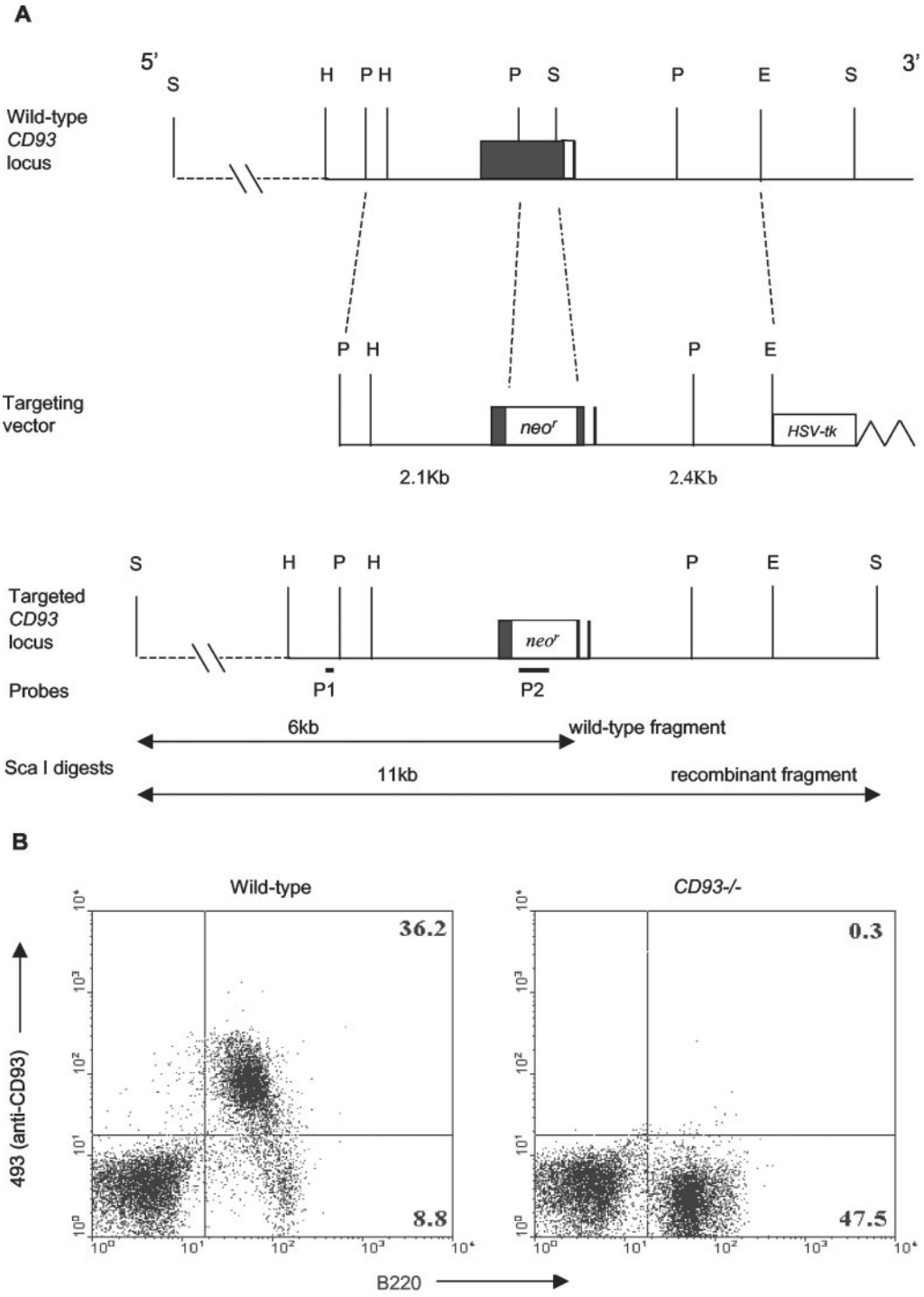

FIGURE 1. Targeted disruption of the murine *CD93* gene. *A*, Schematic representation showing the *CD93* locus, the targeting vector, and the structure of the targeted gene. Filled boxes denote exons and the dotted lines delineate the regions of homology between the wild-type locus and the targeting vector. The neomycin resistance gene (*neo*^{r}) and HSV thymidine cassette (*HSV-tk*) are indicated. Restriction sites are: S, *Sca*I; E, *Eco*RI; P, *Pst*I; H, *Hind*III. The double-headed arrows indicate the size fragments seen in Southern blot analysis of wild-type and recombinant alleles after *Sca*I digestion of genomic DNA hybridized with the 5′external probe (P1). *B*, FACS analysis of CD93 expression on leukocytes (gated by forward and side scatter profiles) from bone marrow of 6-wk-old *CD93*$^{-/-}$ and wild-type mice. B cells were identified using B220 staining (*x*-axis) and CD93 expression was detected using the biotinylated 493 mAb anti-mouse CD93 (*y*-axis). No CD93 staining was detected on immature B cells (B220^{low}) from *CD93*$^{-/-}$ animals confirming the absence of the protein in the targeted mice.

1 h on slides coated with or without C1q and then incubated with fluorescent-labeled mouse RBC coated with IgG2a or C3. The uptake of coated erythrocytes was assessed after a 30-min incubation. In these experiments, we were able to confirm the phenomenon that macrophages cultured on C1q have an enhanced uptake of RBC via both FcγR- and complement-dependent phagocytic pathways compared with macrophages cultured on uncoated plastic (25) (Fig. 3). However, the phagocytic ability of the macrophages was clearly unaffected by the absence of CD93 expression (FcγR-dependent uptake on uncoated slides: 8.93 ± 2.2% by wild-type macrophages compared

Table I. *FACS analysis of cell populations in CD93$^{-/-}$ and wild-type mice*[a]

Compartment	Cell Marker	Predominant Cell Type	CD93$^{-/-}$ Mice	Wild-Type Mice
Bone marrow	CD43$^+$B220$^+$	Pro B	11.5 ± 1.7	11.0 ± 2.6
	B220$^+$IgM$^-$	Pre B$^-$	28.4 ± 4.3	32.0 ± 7.4
	IgM$^+$IgD$^-$	Immature B	15.2 ± 6.5	17.5 ± 7.0
	IgM$^+$IgD$^+$	Mature B	8.7 ± 1.9	8.5 ± 1.2
	7/4$^+$	Neutrophils	20.2 ± 9.9	16.9 ± 11.0
Thymus	CD4$^+$/CD8$^+$	T	62.6 ± 3.3	53.3 ± 8.8
	CD4$^+$	T	21.8 ± 5.7	24.2 ± 16.2
	CD8$^+$	T	8.6 ± 3.4	14.4 ± 11.1
Spleen	B220$^+$	B cells	46.9 ± 10.1	50.6 ± 10.6
	CD4$^+$	T	20.3 ± 6.0	21.3 ± 5.4
	CD8$^+$	T	9.6 ± 4.0	9.8 ± 3.5
	F4/80$^+$	Mac/Eos/DC	4.6 ± 2.6	4.4 ± 3.0
	7/4$^+$	Neutrophils	7.9 ± 3.5	8.6 ± 5.2
	DX-5$^+$	NK/T	5.4 ± 0.5	4.9 ± 1.2
Lymph nodes	B220$^+$	B cells	29.6 ± 5.9	28.6 ± 12.8
	CD4$^+$	T	47.3 ± 4.8	51.6 ± 11.1
	CD8$^+$	T	17.1 ± 6.0	15.0 ± 6.2
Peripheral blood	B220$^+$	B cells	29.2 ± 8.0	25.8 ± 12.5
	CD90.2$^+$	T cells	26.5 ± 8.4	22.2 ± 7.9
	F4/80$^+$	Monocyte/Eos	6.8 ± 2.7	6.3 ± 3.5
	7/4$^+$	Neutrophils	25.1 ± 7.2	32.2 ± 12.7
Peritoneum	B220$^+$	B cells	26.4 ± 8.3	17.7 ± 3.9
	CD90.2$^+$	T cells	9.7 ± 3.2	11.4 ± 5.5
	F4/80$^+$	Macrophages	35.3 ± 11.0	35.7 ± 6.8
	7/4$^+$	Neutrophils	4.3 ± 1.3	4.9 ± 1.4

[a] Cellular composition of CD93$^{-/-}$ and control bone marrow, thymus, spleen, lymph nodes, peripheral blood, and peritoneum. Four to eight mice of each strain were examined at 3 mo of age. For all compartments, the percentages shown represent the percentages of all leukocytes present in that compartment. Total cell counts in all tissues were the same in CD93$^{-/-}$ and wild-type mice. Shown values represent mean ± SEM.

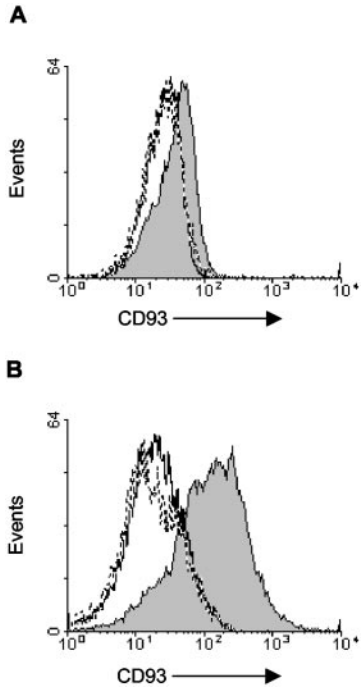

FIGURE 2. Example of flow cytometric histograms comparing CD93 expression between wild-type and CD93-deficient peritoneal macrophages. *A*, Basal CD93 expression on wild-type resident peritoneal macrophages (filled histogram) and *CD93*$^{-/-}$ mice (open histogram). Wild-type resident peritoneal macrophages stained with an irrelevant Ab (dotted line) were also used as a negative control. *B*, CD93 expression on 4-day thioglycolate-elicited wild-type peritoneal macrophages is markedly increased (filled histogram). Thioglycolate-elicited macrophages from *CD93*$^{-/-}$ mice (open histogram) and thioglycolate-elicited wild-type macrophages stained with an irrelevant Ab (dotted line) were used as negative controls. Macrophages were identified by FACS by forward and side scatter profiles and by double staining with F4/80 and anti-CD11b Ab. Data are presented from a single experiment but are representative of results obtained from at least three separate experiments.

with 9.96 ± 2.7% by CD93-deficient macrophages, $p = 0.79$; FcγR-dependent uptake on C1q-coated slides: 16.21 ± 4.3% by wild-type macrophages compared with 18.49 ± 1.3% by CD93-deficient macrophages, $p = 0.64$; complement-dependent uptake on uncoated slides: 5.54 ± 0.88% by wild-type macrophages compared with 7.01 ± 1.14% by CD93-deficient macrophages, $p = 0.35$; complement-dependent uptake on C1q-coated slides: 12.43 ± 1.52% by wild-type macrophages compared with 10.15 ± 0.64% by CD93-deficient macrophages, $p = 0.22$), indicating that under these experimental conditions CD93 does not appear to be a receptor mediating the C1q-dependent effect of enhancement of phagocytic uptake.

FcγR- or complement-mediated phagocytosis in vivo

Although the in vitro assays suggested that CD93 does not contribute to phagocytosis under the conditions used, we explored this further in vivo. We established peritoneal models of clearance of murine RBC precoated in vitro with IgG1 or IgM as described in *Materials and Methods*. The RBCs precoated with IgM when injected into the peritoneum activated the complement system and became opsonized with C3 (data not shown). Fcγ chain-deficient mice, lacking FcγRI and FcγRIII, and C1q-deficient mice were used as negative or positive controls to validate the specificity of the respective assays. Guided by our previous experience, we analyzed cellular uptake 30 min after the peritoneal injection of RBC. Phagocytosis was assessed by FACS (see *Materials and Methods*) and on coded cytospin preparations and the results found to correlate closely. In both experimental models, the phagocytic ability of the *CD93*$^{-/-}$ animals was not significantly different from the strain-matched controls (FcγR-dependent clearance: 24.0 ± 1.0% phagocytic uptake in the *CD93*$^{-/-}$ mice compared with 24.0 ± 3.1% in wild-type controls, $n = 7$ in each group, $p = 0.90$, Mann-Whitney *U* test, and 2.77 ± 0.80% uptake in the Fcγ chain-deficient mice; complement-dependent clearance: 46.0 ± 3.3%, $n = 8$, and 41.4 ± 4.1%, $n = 5$, for *CD93*$^{-/-}$ and wild-type animals, respectively, $p = 0.2977$, and 2.08 ± 0.34% for the C1q-deficient

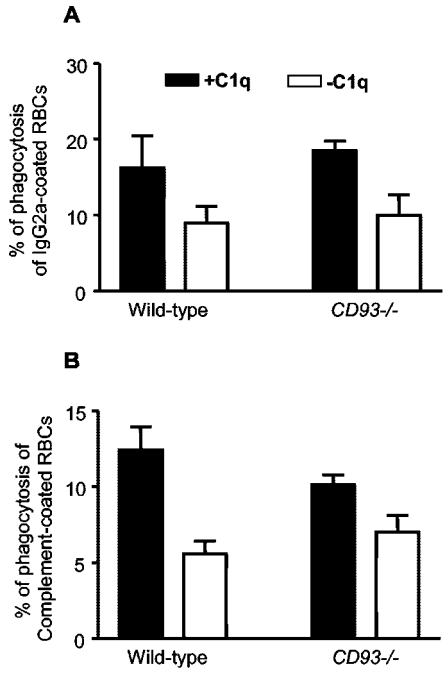

FIGURE 3. C1q-mediated enhancement of phagocytosis of murine RBC in vitro. *A*, FcγR-dependent phagocytosis of murine RBCs coated with a murine IgG2a anti-mouse RBC Ab (34-3C). The graph depicts one experiment with a high level of IgG2a opsonization (10 μg/ml). Similar results were obtained using 1 μg/ml or 100 ng/ml IgG2a 34-3C Ab. *B*, Complement-dependent uptake of murine RBCs opsonized with subagglutinating amount of C3. In both assays, C1q-coated wells were able to induce an increase in phagocytic uptake; however, the lack of CD93 had no effect on this phenomenon. The error bars represent SEM. Each graph represents one experiment that has been repeated three times. Mice used in these experiments were on a C57BL/6 genetic background.

mice), suggesting that this molecule does not provide an important contribution to these phagocytic pathways.

In vivo clearance of apoptotic cells

Recently C1q has been shown to bind directly to apoptotic cells (26) and to mediate the engulfment of these cells by thioglycolate-elicited peritoneal macrophages (20). Because our findings could not rule out the possibility of a role for CD93 in phagocytosis and an interaction with C1q, we decided to explore whether this molecule is involved in the removal of apoptotic cells. We performed the same apoptotic cell clearance assays we originally established in our laboratory for the study of the C1q-deficient mice (20). Apoptotic human Jurkat T cells or apoptotic murine thymocytes, prepared as described in *Materials and Methods*, were injected i.p. in *CD93*$^{-/-}$ mice or strain-matched wild-type controls 4 days after the induction of sterile peritonitis with thioglycolate. The lack of CD93 did not affect either the number of macrophages recruited into the peritoneum compared with strain-matched controls in the three genetic backgrounds analyzed (129/Sv or C57BL/6 or 129/Sv × C57BL/6) (data not shown) or the level of expression of other receptors tested (CR3, CR4, CR1/CR2, FcγRs, and CD14) (data not shown). Guided by our previous experience, the phagocytic uptake was measured 20 min after the injection of the apoptotic cells. In these assays, irrespective of the genetic background of the animals and of the nature of the apoptotic cells used, the *CD93*$^{-/-}$ mice showed a phagocytic defect resulting in a significant decrease in the percentage of macrophages ingesting apoptotic cells assessed by FACS or counting coded cytospin preparations (apoptotic Jurkat T cells: *CD93*$^{-/-}$ 13.8 ± 1.0% compared with wild-type controls 22.8 ± 0.9%, mean ± SEM, $n = 7$ in each group, $p = 0.0006$, Mann-Whitney U test; apoptotic murine thymocytes: 23.7 ± 2.6% and 42.1 ± 2.9% respectively for *CD93*$^{-/-}$ and wild-type animals, $n = 5$ in each group, $p = 0.0079$) (Fig. 4). Although these experiments demonstrated that the CD93 molecule might contribute to the phagocytic clearance of apoptotic cells by inflammatory macrophages in vivo, they did not explore whether this effect was mediated by binding to C1q. To address this, we conducted in vitro phagocytosis assays of apoptotic cells. In these assays, differently from the in vivo experiments where the peritoneal cavity provides the soluble mediators, including complement components, involved in the recognition/uptake of apoptotic cells, the cells were preopsonized with serum in the presence or in the absence of C1q.

In vitro binding and phagocytosis of apoptotic murine thymocytes by thioglycolate-elicited peritoneal macrophages

We initially assessed the level of CD93 expression on wild-type thioglycolate-elicited peritoneal macrophages before and after overnight incubation on plastic wells and demonstrated that the expression was not altered by the overnight culture (data not shown). The phagocytic uptake of apoptotic murine thymocytes was measured after a 20-min incubation in the presence of wild-type or C1q-deficient serum. Surprisingly, there was no significant difference between the phagocytic uptake by CD93-deficient and wild-type macrophages incubated with apoptotic thymocytes preopsonized with wild-type serum or C1q-deficient serum. The uptake by wild-type and *CD93*$^{-/-}$ macrophages was 23.87 ± 2.03%

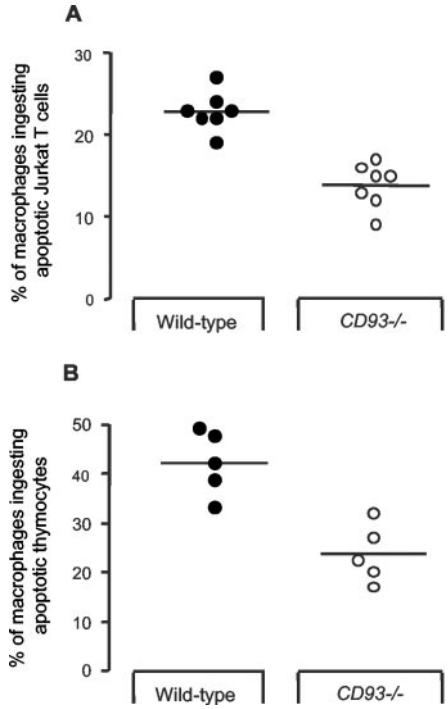

FIGURE 4. Phagocytic clearance of apoptotic cells during sterile peritonitis. Scatter plots showing the percentage of elicited peritoneal macrophages containing phagocytosed apoptotic bodies 20 min after injection of apoptotic Jurkat T cells (*A*) or murine thymocytes (*B*). The *CD93*$^{-/-}$ mice showed a significant reduction in the phagocytosis of apoptotic bodies compared with strain-matched controls ($p = 0.0006$ and $p = 0.0079$ for Jurkat T cells and murine thymocytes, respectively, Mann-Whitney test). Horizontal bars denote means. Data presented are representative of three or more independent experiments. The mice used for the experiment depicted in the graph were on the 129/Sv genetic background. Similar results were obtained using mice on the 129/Sv × C57BL/6 or C57BL/6 genetic backgrounds.

and 24.52 ± 2.58%, respectively ($n = 7$ in each group, $p = 1.000$, Mann-Whitney U test) if the cells were incubated with apoptotic thymocytes preopsonized with wild-type serum and 14.12 ± 1.97% and 14.34 ± 1.98%, respectively ($n = 7$ in each group, $p = 0.9015$, Mann-Whitney U test) when C1q-deficient serum was used (Fig. 5). However, it is of note that in the presence of C1q-deficient serum a significant impairment in the phagocytic uptake by both types of macrophages was observed when compared with wild-type serum (wild-type macrophages: 23.87 ± 2.03% and 14.12 ± 1.97% for thymocytes preopsonized with wild-type or C1q-deficient serum, respectively, $n = 7$ in each group, $p = 0.0175$; *CD93*$^{-/-}$ macrophages: 24.52 ± 2.58% and 14.34 ± 1.98%, respectively, $n = 7$ in each group, $p = 0.0175$, Mann-Whitney U test) (Fig. 5). These findings confirmed the role of C1q as an important soluble mediator involved in the recognition/uptake of apoptotic cells; however, they failed to show a functional interaction between C1q and CD93 in the context of apoptotic cells clearance in vitro. To rule out the possibility that complement production by macrophages during the incubation period may have masked a nonopsonic recognition of apoptotic cells by CD93, we next examined the binding of apoptotic cells to macrophages kept on ice. As expected, cooling the cells at 4°C totally inhibited the uptake of apoptotic cells; nevertheless, there were no detectable differences between the number of cells bound on the surface of the wild-type and *CD93*$^{-/-}$ macrophages (24.83 ± 5.41% and 27.60 ± 3.20%, respectively, $n = 4$, $p = 0.7063$, unpaired t test).

Leukocyte migration in vivo

Previous studies characterizing the mouse homologue of human CD93, known as AA4, suggested an additional function for this molecule besides phagocytosis (13). Because a high expression of this molecule was observed on vascular endothelial cells during embryonic development and angiogenesis, several groups suggested that CD93 might play a role as a homing receptor involved in intercellular adhesion (7, 13). To explore this putative role for CD93, we studied leukocyte recruitment into the peritoneum using three different inflammatory stimuli (thioglycolate, zymosan, and IL-1β). After initial time course experiments, we selected 4 h as the optimal time point for assessing the influx of neutrophils. Human peripheral blood neutrophils were previously shown to express CD93 (11) and we confirmed this observation in mice (data not shown). Irrespective of the inflammatory stimulus used, the numbers and the type of cells recruited into the peritoneal cavity of the *CD93*$^{-/-}$ mice and the strain-matched controls were similar (Table II). In addition, analysis of leukocyte behavior in the cremasteric microcirculation, as observed by intravital microscopy, failed to show any difference in leukocyte rolling flux (data not shown), firm adherence, or transmigration in the *CD93*$^{-/-}$ mice compared with the controls in response to IL-1β (Fig. 6). Overall

Table II. *Peritoneal neutrophil recruitment*[a]

Stimulus	Wild-Type Mice ($\times 10^6$)	CD93$^{-/-}$ Mice ($\times 10^6$)	p Value
IL-1β	8.338 ± 0.776 ($n = 6$)	8.262 ± 0.938 ($n = 6$)	$p = 1.000$
Zymosan A	12.54 ± 2.43 ($n = 5$)	14.54 ± 3.631 ($n = 4$)	$p = 0.9048$
Thioglycolate	9.027 ± 0.468 ($n = 3$)	9.413 ± 0.220 ($n = 3$)	$p = 0.456$

[a] Neutrophil recruitment into the peritoneum of CD93$^{-/-}$ and control mice 4 h postinjection of different inflammatory stimuli. Shown values represent mean ± SEM. The Student t test was used to compare the results.

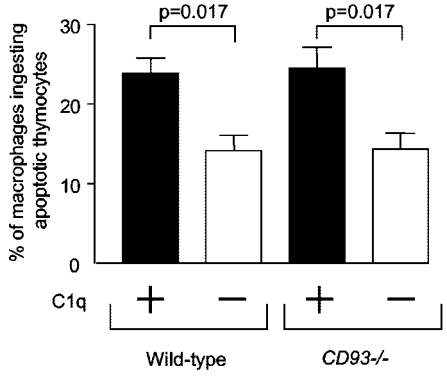

FIGURE 5. Uptake of apoptotic thymocytes by macrophages in vitro. Elicited peritoneal macrophages from *CD93*$^{-/-}$ and control mice were cultured in 24-well plates overnight at a concentration of 0.5×10^6cells/well. Apoptotic murine thymocytes (1×10^6) were then added to the wells in the presence of normal or C1q-deficient serum and the uptake was assessed after 20 min by counting coded cytospin slides. Macrophages from *CD93*$^{-/-}$ and wild-type animals exhibited a markedly impaired uptake in the absence of C1q in the serum; however, the uptake was not different between the two experimental groups irrespective of the type of serum used for preopsonizing the apoptotic cells. The error bars denote SEM. Data shown are representative of three independent experiments.

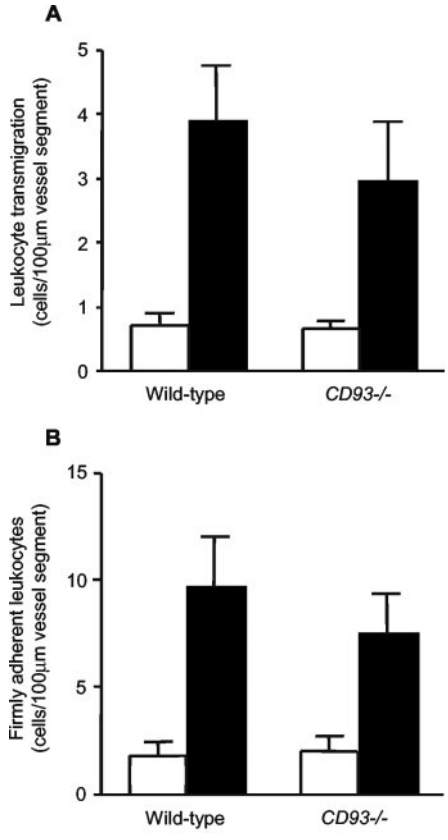

FIGURE 6. Leukocyte responses induced by IL-1β in the cremasteric microcirculation of *CD93*$^{-/-}$mice. *CD93*$^{-/-}$ and wild-type control mice were treated by intrascrotal injection, with either saline (□) or 30 ng of IL-1β both in 400-μl volumes, (■) 4 h before the cremaster muscle was prepared for intravital microscopy. Graphs show leukocyte firm adhesion (*A*) and transmigration (*B*) from the same experiments. No significant differences in leukocyte transmigration or firm adhesion were detected between the two experimental groups. The data represent mean ± SEM ($n = 4$ per group).

these findings indicate that the lack of CD93 expression on endothelial cells does not appear to have a marked effect on the intercellular adhesion mechanisms involved in mediating leukocyte migration in the models used.

Discussion

Human C1qRp, originally identified as a protein that mediates the ability of C1q to enhance phagocytosis in vitro, has recently been identified as CD93 and its binding to C1q has been questioned (9, 10). In this study, we used gene-targeted CD93-deficient mice to present several lines of evidence indicating that the mouse homologue of the human CD93 is not required for the C1q-mediated events seen in complement- and FcγR-dependent phagocytic assays. However, an impaired uptake of apoptotic cells in vivo was detected in the CD93-deficient mice suggesting that this receptor may contribute to the removal of dying cells.

Studies have shown that C1q is able to interact with various cell types and trigger numerous biological responses (reviewed in Ref. 1). However, the identification of the molecule(s) interacting with C1q on the cell surface to initiate these responses has proved difficult. A number of putative candidates have been described in the literature (27), but their nature as specific C1q receptors is still controversial. Among these candidate CD93 was shown to be a type I transmembrane protein containing an N-terminal region with homology to C-type lectin domains and a short cytoplasmic tail. The initial characterization of this molecule by Tenner and coworkers (4) demonstrated that some Abs directed against CD93 were able to inhibit the C1q-dependent enhancement of phagocytosis suggesting that CD93 is involved, maybe as part of a signaling complex, in these C1q-mediated events. However, in these early studies there was no clear demonstration of a direct interaction between C1q and CD93. In contrast, two recent studies, using CD93-transfected cells, have provided evidence for the lack of C1q binding to CD93 (9, 10) under physiological conditions. In this study, we investigated this controversial interaction using macrophages obtained from CD93-deficient mice. Although in our assays we were able to reproduce the enhancement of FcγR- and complement-dependent uptake in macrophages plated on C1q-coated wells, as originally reported (25), the lack of CD93 expression had no effect on this phenomenon. In support of these observations in vitro, we showed that complement- or FcγR-dependent uptake by peritoneal macrophages in vivo was not impaired in the CD93-deficient animals compared with the wild-type mice. Thus our findings, taken together, indicate that CD93 does not play a key role in the C1q-mediated effects on phagocytosis and provide support to the recent observations that CD93 may not be a true C1q receptor. Can these findings be reconciled with the initial observations reported by Tenner and colleagues (4)? It is possible that blocking CD93 with Abs might have attenuated the C1q-mediated events in a manner that did not depend on interfering with C1q binding. If CD93 is part of a complex of proteins on the cell surface involved in phagocytosis, it is conceivable that removal of one protein of the complex may cause less functional disruption than the interference caused by the binding of an Ab to the protein.

Although the biological role of CD93 is still unclear, its cellular expression is becoming less controversial. The initial studies by Tenner and colleague (11) showed that in humans, this molecule is present on cells of myeloid lineage, endothelial cells, and platelets. Follow-up studies by the same group on the murine homologue revealed a similar expression profile (6). More recent studies, using a wide range of Abs, found that CD93 is not expressed by tissue macrophages, even in inflamed tissue, and is predominantly present on endothelial cells (7, 13–15). In addition, its expression on peripheral blood monocytes was shown to decrease in culture when the cells were differentiated into dendritic cells (15). In agreement with some of the previous observations (8, 15), we detected a low CD93 expression on murine peripheral blood neutrophils and resident peritoneal macrophages. Interestingly, a marked increased expression after thioglycolate elicitation was also observed suggesting that the expression of this molecule is tissue specific and/or can be influenced by the activation state of the macrophages. Taken together, our data provided novel contributions to the characterization of the cellular expression of CD93 and how it may be regulated in response to different inflammatory stimuli.

The predominant location of CD93 on vascular endothelial cells, its tightly regulated expression on hemopoietic cells and endothelial cells during embryogenesis, and the presence of similar motifs shared by other intercellular adhesion molecules have led to speculations that this receptor may play a role in cell-cell interaction and in angiogenesis (7, 9, 13, 14). We tested these hypotheses in vivo by examining whether the lack of CD93 during development might have caused changes in cellular distribution or altered the capacity of peripheral blood cells to adhere and transmigrate in response to inflammatory stimuli. The full viability of the CD93-deficient mice clearly indicate that the deletion of the CD93 gene has no detrimental effects on the development of the mice that survived normally in specific pathogen-free conditions up to 1 year of age (data not shown). Consistent with this observation, we found no obvious abnormalities in the percentage or in the phenotype of the cell populations present in all tissues analyzed. In addition, cell recruitment of neutrophils to the peritoneum, induced by three different inflammatory stimuli, was not impaired in the CD93-deficient mice. Similarly, there were no differences in leukocyte adhesion and transmigration in the cremasteric microcirculation in response to locally administered IL-1β, as detected by intravital microscopy. These results show that CD93 does not play a key role in cell-cell interaction leading to leukocyte migration in these experimental models. However, cell-cell interactions are known to be complex and regulated by multiple molecules and it is possible that, under our experimental conditions, other receptors may have been recruited that were able to functionally compensate for the absence of CD93. Future work will be required to fully elucidate this aspect.

It is now widely accepted that C1q can bind to apoptotic cells (26, 28) and plays a role in the clearance of apoptotic bodies in vivo (20). Given the role, although controversial, of CD93 as a putative C1q receptor with phagocytic properties, we decided to explore whether this molecule was involved in the removal of apoptotic cells. Using the peritonitis model established in our laboratory (20), we showed that the *CD93*$^{-/-}$ mice had a significantly impaired uptake of apoptotic cells by thioglycolate-elicited macrophages compared with strain-matched controls. The in vivo phagocytic defect was confirmed using *CD93*$^{-/-}$ mice on three different genetic backgrounds and using two sources of apoptotic cells (Jurkat T cell and murine thymocytes). Although the in vivo experiments suggested a role for CD93 in the removal of dying cells, the in vitro findings did not provide supporting evidence. After an overnight culture on plastic wells, the *CD93*$^{-/-}$ macrophages failed to show a reduction in the uptake or in the binding of apoptotic cells when compared with wild-type macrophages. The explanation for the differences between the in vivo and the in vitro data may lie in the redundancy and complexity of the clearance systems involved in the recognition and engulfment of apoptotic cells by macrophages (reviewed in Refs. 29 and 30). As suggested by studies on receptors for which there is in vitro but not in vivo evidence of involvement in apoptotic cell uptake (31), the mechanisms by which apoptotic cells are identified, taken up, and

degraded by phagocytes may depend on the experimental conditions applied. We observed no apparent functional compensation for the absence of CD93 in the uptake of apoptotic cells in vivo, but the data demonstrated that other receptors could do so in vitro. One may speculate that in the in vivo model the macrophages and apoptotic cells were in the fluid phase with the macrophages encountering the apoptotic cells in suboptimal circumstances for their clearance. Under these conditions, CD93, probably as part of a complex of phagocytic proteins expressed on the cell surface of macrophages, may play an important role in the recognition/engulfment of dying cells. In the in vitro experiments, the apoptotic cells are spun down onto the macrophages adhered to plastic and it is possible that under these conditions other receptors may have been recruited that were able to fully compensate for the absence of CD93. It is of note that both wild-type and CD93-deficient peritoneal macrophages exhibited a markedly reduced engulfment of apoptotic cells in the absence of C1q in the medium. Interestingly, the CD93-deficient mice, unlike the C1q-deficient mice (22), did not develop features of a lupus-like disease as assessed by renal histology and anti-ssDNA Abs (data not shown). These findings, taken together, confirmed the role of C1q as an important soluble bridge molecule involved in the removal of dying cells and provided further indirect support to the hypothesis that CD93 does not interact with C1q. Indeed, a recent publication using Abs to various potential C1q receptors suggested that it is the cC1qR (collagenous tail C1q receptor) in cooperation with CD91 and not CD93 that is important in the C1q-dependent phagocytosis of apoptotic cells by human monocyte-derived macrophages in vitro (32).

In summary, we have generated mice lacking CD93 and demonstrated that these mice have a phagocytic defect in the uptake of apoptotic cells in vivo indicating that CD93 might play a contributing role in the removal of dying cells. We have also shown that CD93 does not contribute to the C1q-dependent enhancement of phagocytosis in vitro and most likely does not function as a C1q receptor. In addition, in the *CD93*$^{-/-}$ mice we found no experimental evidence to support a role for this protein as an intercellular adhesion molecule. Further studies will be needed to understand the biological relevance of these novel observations.

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