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Suzanne S. Bohlson, Richard Silva, Maria I. Fonseca and Andrea J. Tenner

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CD93 Is Rapidly Shed from the Surface of Human Myeloid Cells and the Soluble Form Is Detected in Human Plasma¹

Suzanne S. Bohlson,² Richard Silva, Maria I. Fonseca, and Andrea J. Tenner

CD93 is a highly glycosylated transmembrane protein expressed on monocytes, neutrophils, endothelial cells, and stem cells. Antibodies directed at CD93 modulate phagocytosis, and CD93-deficient mice are defective in the clearance of apoptotic cells from the inflamed peritoneum. In this study we observe that CD93, expressed on human monocytes and neutrophils, is susceptible to phorbol dibutyrate-induced protein ectodomain shedding in a time- and dose-dependent manner. The soluble fragment found in culture supernatant retains the N-terminal carbohydrate recognition domain and the epidermal growth factor repeats after ectodomain cleavage. Importantly, a soluble form of the CD93 ectodomain was detected in human plasma, demonstrating that shedding is a physiologically relevant process. Inhibition of metalloproteinases with 1,10-phenanthroline inhibited shedding, but shedding was independent of TNF- α -converting enzyme (a disintegrin and metalloproteinase 17). Phorbol dibutyrate-induced CD93 shedding on monocytes was accompanied by decreased surface expression, whereas neutrophils displayed an increase in surface expression, suggesting that CD93 shed from the neutrophil surface was rapidly replaced by CD93 from intracellular stores. Cross-linking CD93 on human monocytes with immobilized anti-CD93 mAbs triggered shedding, as demonstrated by a decrease in cell-associated, full-length CD93 concomitant with an increase in CD93 intracellular domain-containing cleavage products. In addition, the inflammatory mediators, TNF- α and LPS, stimulated ectodomain cleavage of CD93 from monocytes. These data demonstrate that CD93 is susceptible to ectodomain shedding, identify multiple stimuli that trigger shedding, and identify both a soluble form of CD93 in human plasma and intracellular domain containing cleavage products within cells that may contribute to the physiologic role of CD93. *The Journal of Immunology*, 2005, 175: 1239–1247.

Protein ectodomain cleavage, or shedding, is a common feature of adhesion molecules that is generally mediated by metalloproteases (1). Shedding modulates homing properties of activated leukocytes (2) and releases bioactive ectodomain products. For example, soluble TNF- α , TGF- α , TGF- β , and members of the epidermal growth factor (EGF)³ family are all derived from shedding of transmembrane proteins (3). Another consequence of shedding is the release of the intracellular domain (ICD) fragment after regulated cleavage of the transmembrane domain (4). The ICD fragment of Notch, CD44, and others relocates to the nucleus and regulates gene transcription (5). Therefore, shedding affects numerous aspects of cell biology and subsequent physiology via both intracellular and extracellular pathways.

Shedding is largely mediated by zinc-dependent metalloproteinases of the matrix metalloproteinase, membrane type-matrix metalloproteinase or a disintegrin and metalloproteinase (ADAM) family. TNF- α -converting enzyme (TACE) (ADAM17) is the

most widely investigated sheddase and is responsible for cleavage of numerous transmembrane proteins, including L-selectin, TNF- α , TGF- α , TGF- β , members of the EGF family, and the amyloid precursor protein (6). The majority of mice made deficient in TACE die between embryonic day 17.5 and the first day after birth, illustrating the importance of ectodomain shedding in mammalian development (7). However, TACE is not a universal sheddase, because a variety of transmembrane proteins, including CD44, are cleaved by distinct metalloproteinases (8).

CD93 (C1qR_p, AA4.1 (mouse)) is a 100,000 *M_r* type 1 transmembrane glycoprotein that has been implicated in the regulation of cell-cell interactions during development and in the efficiency of phagocytosis (9–12). Mice deficient in CD93 display an impairment in the uptake of apoptotic cells *in vivo*. In addition, an IgM anti-CD93 mAb (R3) triggers phagocytosis when cells are plated on the immobilized Ab and inhibits phagocytosis when cells are treated in the fluid phase (13). However, the mechanisms responsible for these effects on phagocytosis are unknown (10). CD93 has been implicated in regulating adhesive processes, because ligation of CD93 with the F(ab')₂ of the mAb mNI-11 stimulates homotypic aggregation of LPS-stimulated U937 cells, and homotypic aggregation is inhibited by Abs against LFA-1 or ICAM-1 (14). The same Ab triggers rapid spreading of HUVEC cells when immobilized on a surface, and this spreading is inhibited by cytochalasin D (such spreading is not detected with control samples after HUVEC exposure to anti-CD44) (15). CD93 is expressed on the endothelium and on circulating myeloid cells and platelets, consistent with a role for CD93 in cellular homing to sites of inflammation (12, 16). Furthermore, CD93 expression defines the earliest human bone marrow stem cells because it is expressed on CD34-negative and -positive hemopoietic and hepatic precursors, and CD93 is a common marker for murine B cell development (17, 18).

Department of Molecular Biology and Biochemistry, Center for Immunology, University of California, Irvine, CA 92697

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² Address correspondence and reprint requests to Dr. Suzanne S. Bohlson, Department of Molecular Biology and Biochemistry, University of California, 2419 McGaugh Hall, Irvine, CA 92697. E-mail address: sbohlson@uci.edu

³ Abbreviations used in this paper: EGF, epidermal growth factor; GalNAc, *N*-acetyl galactosamine; HSA, human serum albumin; ICD, intracellular domain; PAF, platelet-activating factor; PDBu, phorbol dibutyrate; TAPI, TNF- α protease inhibitor; PVDF, polyvinylidene difluoride; sCD93, soluble CD93; TACE, TNF- α -converting enzyme; ADAM, a disintegrin and metalloproteinase.

Previous studies demonstrated that glycosylation of CD93 stabilized surface expression and that under conditions of induced hypoglycosylation, increased levels of CD93 were detected in culture medium, suggesting that CD93 was proteolytically cleaved from the surface (19). Because shedding has been implicated as an important mechanism for regulation of other adhesion molecules with structural and functional similarities to CD93 (e.g., L-selectin (2) and CD44 (8)), we chose to investigate cell stimuli that might induce CD93 shedding. The work described in this study shows that CD93 is susceptible to metalloproteinase-mediated shedding induced by the phorbol ester, phorbol dibutyrate (PDBu), independent of the glycosylation state. Interestingly, surface cleavage of CD93 occurs in both human monocytes and neutrophils, but surface CD93 expression is actually increased in neutrophils, possibly due to an intracellular store of CD93 within neutrophil granules. Importantly, soluble CD93 (sCD93) was detected in normal human plasma, suggesting that the cleavage event is physiologically relevant, and cytoplasmic tail cleavage products were detected in cell lysates, indicating that the liberated CD93-ICD may also have biologic activity. In addition, CD93 shedding was induced by cross-linking with mAbs and the inflammatory mediators, TNF- α and LPS, indicating physiologic pathways that trigger this event. These data demonstrate a novel mechanism of regulated expression of CD93 that may have implications in stem cell development and inflammation.

Materials and Methods

Reagents and Abs

R139 and R3 were generated by immunization with C1q-binding proteins isolated from U937 cell extracts as previously described (13, 20). The polyclonal anti-CD93 cytoplasmic tail Ab 1150 was generated against the C-terminal 11 aa of CD93 as previously described (21). Polyclonal anti-CD93 1157 was generated by immunization with sCD93 produced in insect SF9 cells as previously described (16). The anti-L-selectin mAb, Dreg 56, was purchased from eBiosciences. FITC- or PE-conjugated secondary F(ab')₂ Abs were obtained from Jackson ImmunoResearch Laboratories. TNF- α was purchased from PeproTech. C5a was a gift from R. DiScipio (La Jolla Institute for Molecular Medicine, San Diego, CA). TNF- α protease inhibitor (TAPI) 1 and -2 were obtained from Peptides International. R0-31-9790 and R0-32-7315 were obtained from Hoffmann La Roche. Ultra Pure LPS was purchased from List Biological Laboratories. All media were obtained from Invitrogen Life Technologies unless otherwise noted. All other reagents were purchased from Sigma-Aldrich at the highest quality unless stated otherwise.

Cells and plasma

Human monocytes and lymphocytes were purified by counterflow elutriation using a modification of the technique of Lionetti et al. (22) as described previously (23). Plasma from normal healthy donors was saved at the time of elutriation and centrifuged at 16,000 \times g for 10 min to remove platelets before use. In some experiments, IgG was depleted from plasma by overnight incubation at 4°C with 120 μ l of GammaBind G-Sepharose (Amersham Biosciences) to 200 μ l of plasma. Neutrophils were purified from human blood as previously described (24) and resuspended in HBSS⁺/0.25% human serum albumin (HSA; American Red Cross, distributed by FFF Enterprises) with 20 mM HEPES. *ldlD* cells were a gift from Dr. M. Krieger (Massachusetts Institute of Technology, Cambridge, MA) and were transfected with CD93 (*ldlD*-CD93) as previously described (19). *ldlD*-CD93 were maintained in serum-free medium, Chinese hamster ovary-serum-free medium II, supplemented with 100 U/ml penicillin G sodium/100 μ g/ml streptomycin sulfate (Invitrogen Life Technologies) and 400 μ g/ml G418 sulfate (Cellgro). To reconstitute wild-type glycosylation, 20 μ M galactose and 400 μ M *N*-acetylgalactosamine were added to culture medium 48 h before experiments were conducted. HEK293T cells were cultured in DMEM with 10% FBS (HyClone), 100 U/ml penicillin G sodium/100 μ g/ml streptomycin sulfate (Invitrogen Life Technologies), 1 \times nonessential amino acids (Invitrogen Life Technologies), and 10 mM HEPES, pH 7.4.

Cell activation

For analysis of surface expression by flow cytometry, monocytes were resuspended at 2 \times 10⁶/ml in RPMI 1640/10% heat-inactivated FBS, 10 mM HEPES and stimulated as described in the figure legends. For ELISA

studies, monocytes were resuspended at densities \geq 8 \times 10⁶/ml before activation, and neutrophils were resuspended at \geq 3 \times 10⁷/ml. For immunoprecipitation studies, monocytes were treated in serum-free HL1 medium (BioWhittaker). For inhibitor treatment, 5 mM 1,10-phenanthroline (dissolved in methanol to 2 M) was added immediately before addition of PDBu. For TACE inhibitor studies, inhibitors were added 30 min before addition of PDBu. All TACE inhibitors were dissolved in DMSO, except for TAPI-2, which was dissolved in HL1 medium (BioWhittaker). For Ab cross-linking experiments, Abs were coated on two-well Lab-Tek chamber slides (Nalge Nunc International) at 50 μ g/ml for 2 h in 0.1 M carbonate buffer, pH 9.5. Chamber slides were washed with sterile PBS, and 2 \times 10⁶ cells in 1 ml of RPMI 1640/10% FCS/10 mM HEPES were added, centrifuged at 70 \times g for 3 min, and incubated at 37°C for various time periods. Monolayers were washed with ice-cold PBS, and adherent cells were harvested by scraping in 0.5 ml of extraction buffer (0.3% Nonidet P-40, 10 mM triethanolamine, 1 mM CaCl₂, 1 mM MgCl₂, 0.15 M NaCl, 0.1 M PMSF, and 1/200 protease inhibitor mixture set III (Calbiochem)) on ice. Cell supernatants and wash containing nonadherent cells were spun down at 1,000 rpm for 10 min, washed with ice-cold PBS, and added to the cell lysates. Cells were lysed for 1 h on ice and centrifuged at 14,000 rpm for 15 min at 4°C, and soluble material was analyzed by ELISA or Western blot.

Flow cytometry

Cells (1 \times 10⁶) were washed twice in ice-cold FACS buffer (HBSS⁺, 0.2% BSA, and 0.2% sodium azide) and incubated with primary Abs (3 μ g of IgM/R3 or 1 μ g of IgG1/Dreg 56) for 30 min on ice with shaking every 10 min. Cells were washed twice in ice-cold FACS buffer and incubated with 2 μ l of FITC- or PE-conjugated secondary Abs (F(ab')₂) for 30 min on ice, with shaking every 10 min. Cells were washed three times and either analyzed immediately or fixed overnight in 1% formaldehyde (prepared fresh) before analysis. Analysis was performed using the FACSCalibur apparatus (BD Biosciences) and the CellQuest program.

ELISA

CD93 sandwich ELISA using anti-CD93 mAbs R139 and R3 was performed as previously described (19). To estimate the amount of CD93 in picomolar concentrations, values were compared with a standard curve of U937 cell extract. U937 cells were previously shown to contain 8.1 \times 10⁴ molecules of CD93/cell (19). In some experiments, values in picomolar concentrations were divided by the total number of cell equivalents. To detect recombinant CD93 fusion proteins anti-V5 (Invitrogen Life Technologies) was used at a 1/1000 dilution to capture recombinant CD93 ectodomain fragments, and R3 was used to detect. To determine whether sCD93 contained a cytoplasmic tail, an ELISA was used in which 1150 (rabbit polyclonal anti CD93 cytoplasmic tail) was used at 20 μ g/ml to capture, and R3 was used to detect.

Immunoprecipitation and Western blot

Tissue culture supernatants were precleared for 2 h with 10 μ g of IgG2b (ICN Pharmaceuticals) and 25 μ l of a 1/1 slurry of GammaBind G-Sepharose (Amersham Biosciences). CD93 was immunoprecipitated from precleared tissue culture supernatants by incubation with 10 μ g of R139 for 1 h at 4°C, followed by a similar incubation with 25 μ l of a 1/1 slurry of GammaBind G-Sepharose. The beads were washed three times in extraction buffer (described above) and boiled in nonreduced SDS-PAGE sample buffer. For Western blot analysis, proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). After blocking in 5% milk/TBST (20 mM Tris, 150 mM NaCl, and 0.1% Tween 20), blots were probed for 2 h at room temperature with primary Abs. Blots were washed and probed with secondary HRP-conjugated Abs for 1 h at room temperature and developed using ECL (Amersham Biosciences).

Expression of rCD93 extracellular domains

The CD93-CRD was amplified from pcDNA3.1-CD93 (11) using the following primers: 5'-GGAATTCAGAGGGCCACACAGAGACCG and 3'-GATATCTGAAGCT-GAAGTGCACAC. The CD93 CRD-EGF₃ was amplified using the same 5' primer and 3'-GATATCTGGTGCAGAGACCCC. The endogenous CD93 signal sequence was included in the constructs. The PCR products were subcloned into pGEM-T (Promega) and sequenced at the University of California-Irvine core facility. The products from pGEM-T were cloned into a modified pENTR-11 (Invitrogen Life Technologies) vector that had been engineered to remove its start codon. Target genes were transferred to pcDNA-DEST40 via λ phage site-specific recombination as described by the manufacturer of GATEWAY cloning technology (Invitrogen Life Technologies). HEK 293T cells were transfected with the indicated plasmids or, as a control, full-length CD93 in

pCDNA3.1 using LipofectAMINE (Invitrogen Life Technologies) according to the manufacturer's instruction. Supernatants were used as a source of recombinant proteins. Proteins were purified by nickel chromatography according to the manufacturer's protocol (Invitrogen Life Technologies).

Immunofluorescence and confocal microscopy

Human neutrophils (2×10^5) in 1 ml of HBSS⁺/0.25% HSA/20 mM HEPES were plated on sterilized coverslips in a 12-well plate and incubated at 37°C for 1 h. Cells were washed twice for 5 min each time with PBS and fixed in 3.7% formaldehyde/PBS for 10 min at room temperature. Cells were washed twice more with PBS and incubated overnight at 4°C before staining. For permeabilization, cells were incubated with 0.1% Triton/PBS for 5 min. The immunostaining procedure performed was previously described (16). Briefly, cells (permeabilized and nonpermeabilized) were incubated with 2% BSA/2% normal donkey serum in PBS (blocking solution) for 1 h at room temperature. Next, cells were labeled with either an anti-CD93 rabbit polyclonal Ab (1157) or a rabbit IgG control Ab at 5 μ g/ml in blocking solution (1 h at room temperature), followed by Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories; 1/500 in blocking solution; 1 h at room temperature). Coverslips were mounted with Vectashield (Vector Laboratories). Neutrophils were visualized using a Zeiss LSM 510 META confocal microscope.

Results

CD93 is shed from the surface of PDBu-stimulated monocytes

To determine whether CD93 was susceptible to ectodomain cleavage, human monocytes were treated with increasing concentrations of the phorbol ester PDBu, a known potent inducer of shedding (25). A decrease in cell surface CD93 was detected by flow cytometry with increasing concentrations of PDBu, with a maximum decrease in surface expression at 50 nM PDBu (Fig. 1A). To assess the kinetics of the PDBu-induced decrease in surface expression, monocytes were treated with 50 nM PDBu, and surface expression was assessed at 2, 5, 10, 20, and 60 min. The decrease in cell surface expression was significant at 20 min after addition of PDBu ($p = 0.02$, by paired Student's *t* test) and was also enhanced at 60 min (80.6% shedding; $n = 3$; Fig. 1B). To determine whether sCD93 was present in medium from PDBu-stimulated monocytes, medium was analyzed in a sandwich ELISA using anti-CD93 mAbs R139 and R3. The sCD93 was detected at higher levels (6- to 17-fold) in medium from PDBu-stimulated cells compared with

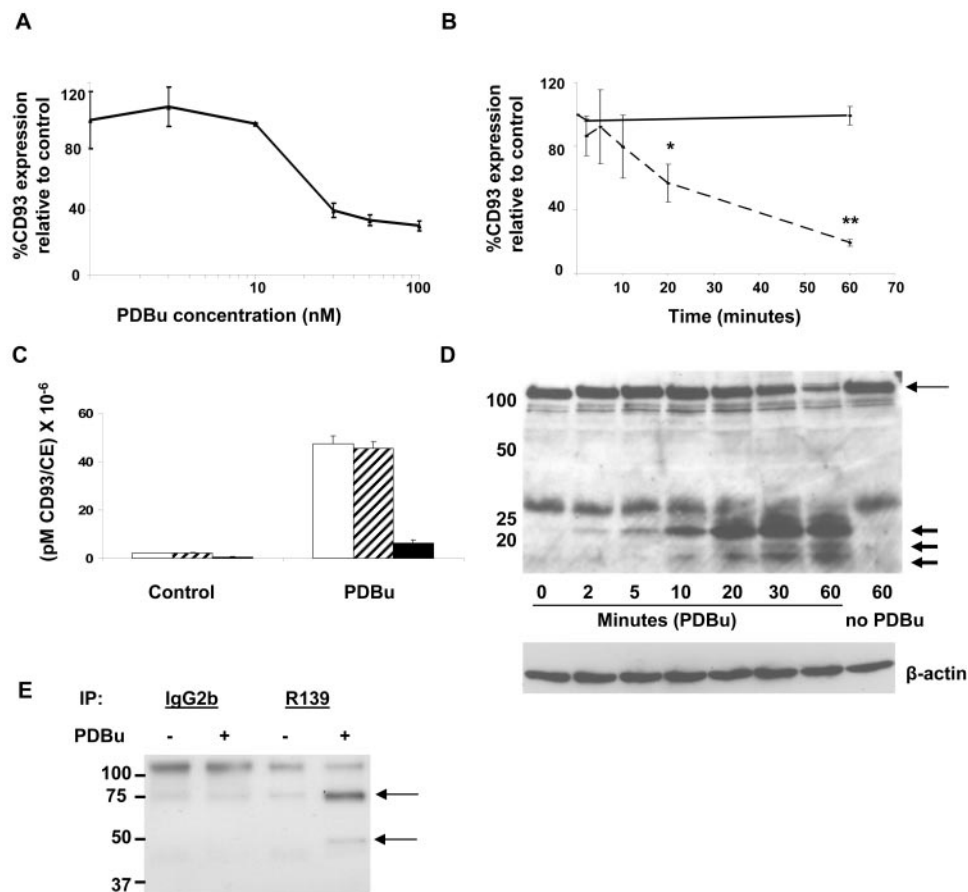


FIGURE 1. CD93 is shed from human monocytes in response to PDBu. *A*, Human monocytes were stimulated with increasing concentrations of PDBu for 30 min at 37°C and analyzed for CD93 expression by flow cytometry. Depicted is the average mean fluorescence of three experiments with SD relative to the untreated control. *B*, Monocytes were treated with 50 nM PDBu for various time periods and analyzed by flow cytometry after staining with anti-CD93 mAb (R3). Depicted is the average mean fluorescence from three experiments (\pm SD). *, $p = 0.02$; **, $p = 0.001$ (by paired Student's *t* test). *C*, Monocytes (1.16×10^6) in 1 ml were treated with 100 nM PDBu for 20 min. Supernatants were left untreated (\square) or were cleared with 5 μ g of anti-CD93 Ab (R139; \blacksquare) or isotype control (IgG2b; \square), followed by protein G-Sepharose and tested for CD93 by ELISA, as described in *Materials and Methods*. Shown is the average of triplicate samples (\pm SD) from one experiment, representative of two. *D*, Equal volumes of cell lysates from human monocytes treated with 50 nM PDBu for increasing amounts of time were separated by 4–20% gradient SDS-PAGE, transferred to PVDF, and probed with 5 μ g/ml polyclonal anti-CD93 cytoplasmic tail Ab 1150. The blot was stripped and reprobed for β -actin to control for equal protein loading. The thin arrow points to full-length CD93. Three thick arrows point to CD93 cytoplasmic tail containing cell-associated fragments running at <25 kDa. *E*, Monocytes (1×10^7) in 500 μ l of serum-free medium (HL1) were treated with or without PDBu for 30 min. Supernatants were immunoprecipitated with IgG2b or R139 and resolved by 8% nonreduced SDS-PAGE, and CD93 was detected by Western blot with 1 μ g/ml biotin R3.

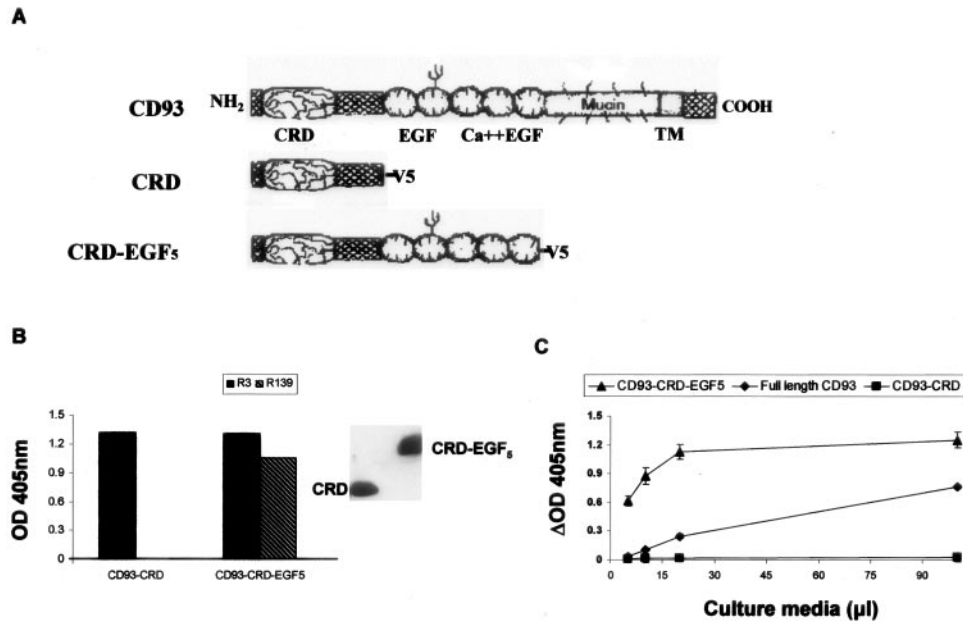


FIGURE 2. R3 recognizes the CD93-CRD, and R139 recognizes the EGF repeat domain of CD93. *A*, Schematic of CD93 and recombinant CD93 extracellular domain fragments generated by expression in HEK293T cells. *B*, Supernatants from HEK293T cells transfected with CD93 fusion proteins were tested by sandwich ELISA using the anti-epitope tag V5 Ab to capture (1/1000) and either R3 (■) or R139 (▨) to detect. The presence of purified recombinant protein domains was verified by Western blot with R3 (5 $\mu\text{g}/\text{ml}$; *inset*). *C*, Supernatants from *B* were tested by sandwich ELISA using the anti-CD93 mAb R139 to capture (1 $\mu\text{g}/\text{well}$) and biotinylated anti-CD93 R3 mAb to detect (2 $\mu\text{g}/\text{ml}$), demonstrating the presence of sCD93 produced constitutively in transfected HEK293T cells and supporting the immunoreactivity seen in *B*.

medium from nonstimulated cells. This activity in the ELISA was depleted when the medium was absorbed with anti-CD93 (R139), but not with the isotype control Ab (IgG2b), verifying that the ELISA was specific (Fig. 1C; $n = 2$). Full-length CD93 expression decreased over time after stimulation with PDBu, whereas a gradual increase in smaller cell-associated fragments containing the CD93 cytoplasmic tail was detected by Western blot (Fig. 1D), indicative of proteolytic processing of CD93. The presence of a soluble form of CD93 in tissue culture supernatants was confirmed by immunoprecipitation of CD93 (using R139) from monocyte supernatants after treatment with 50 nM PDBu. A 75,000 M_r band and a 50,000 M_r band reactive with anti-CD93 (R3; Fig. 1, arrows) were apparent after treatment with PDBu (but not without PDBu; $n = 2$; Fig. 1E).

Soluble CD93 contains both the CRD domain and the EGF repeats

Because both anti-CD93 mAbs R3 and R139 recognized the soluble fragment(s) of CD93 in the culture supernatant from PDBu-stimulated cells, recombinant proteins encoding either the CRD domain of CD93 or the CRD domain followed by the EGF repeats (CRD-EGF₅; Fig. 2A) were expressed and used to map the interaction site of the Abs to determine the nature of the soluble fragment. The proteins were expressed in HEK293T cells, and supernatants were tested by Western blot and ELISA. When a sandwich ELISA was performed using a capture Ab recognizing the V5 epitope tag found on both recombinant fusion proteins, reactivity with R3 was detected for both fusion proteins, whereas reactivity with R139 was detected only with the V5-CRD-EGF₅ fusion protein, indicating that R3 recognizes the CRD domain and R139 recognizes a region within the EGF repeats (Fig. 2B). Furthermore, after purification by nickel chromatography, both proteins were recognized with R3 by immunoblot analysis (Fig. 2B, *inset*). Also, the fusion protein encoding the CRD-EGF₅ displayed reactivity in an R3/R139 sandwich ELISA, whereas no reactivity by ELISA

was demonstrated for the CRD alone (Fig. 2C). These data demonstrate that R3 and R139, Abs that modulate phagocyte activity (20, 26), recognize distinct domains within CD93 and suggest that sCD93 consists of the CD93 N terminus and contains both the CRD and EGF₅ repeats, because the mAbs recognize these distinct protein domains.

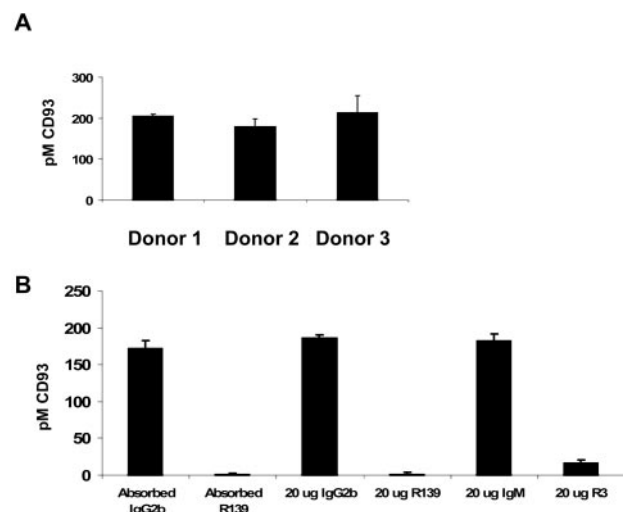


FIGURE 3. Human plasma contains sCD93. *A*, Plasma from three normal human donors was diluted (1/3 or 1/10) and tested for the presence of sCD93 by sandwich ELISA with R139 and R3. The average of triplicate wells (\pm SD) is shown. *B*, Soluble CD93 in plasma was depleted by absorption with 20 μg of R139 or IgG2b coupled to protein G-Sepharose per 100 μl of plasma or was competed by addition of 20 μg of R139 or 20 μg of R3 to 115 μl of plasma before dilution and analysis. The average of triplicate wells (\pm SD) is shown.

Soluble CD93 is detected in human plasma/serum

To determine whether sCD93 was produced *in vivo*, human plasma was analyzed for the presence of CD93 by ELISA. Plasma from three healthy donors contained measurable levels of sCD93, and these levels were similar among the three donors (Fig. 3A). The activity in the ELISA was specific, because it was lost after absorbance with R139, and it was competed by the addition of R139 or R3, but not isotype control Abs (Fig. 3B). Soluble CD93 was also detected in human serum at levels similar to those in plasma (data not shown). The concentration of sCD93 was estimated to be 0.2 nM based on the number of molecules per sample in a standard curve of U937 cell extracts that expresses $\sim 8.1 \times 10^4$ molecules/cell (19). The sCD93 in serum and monocyte cul-

ture medium after PDBu stimulation was not reactive with the anti-CD93 cytoplasmic tail Ab, as determined by ELISA, consistent with ectodomain proteolysis (data not shown).

CD93 shedding is mediated by a metalloproteinase, but is independent of TACE (ADAM17)

Because metalloproteinases are responsible for most of the reported ectodomain cleavage (1), 1,10-phenanthroline, a zinc-chelating compound and inhibitor of metalloproteinases, was tested for its ability to inhibit PDBu-stimulated shedding of CD93. Monocytes treated with PDBu in the presence of 1,10-phenanthroline were completely resistant to PDBu-induced ectodomain cleavage, as assessed by flow cytometry (Fig. 4, A

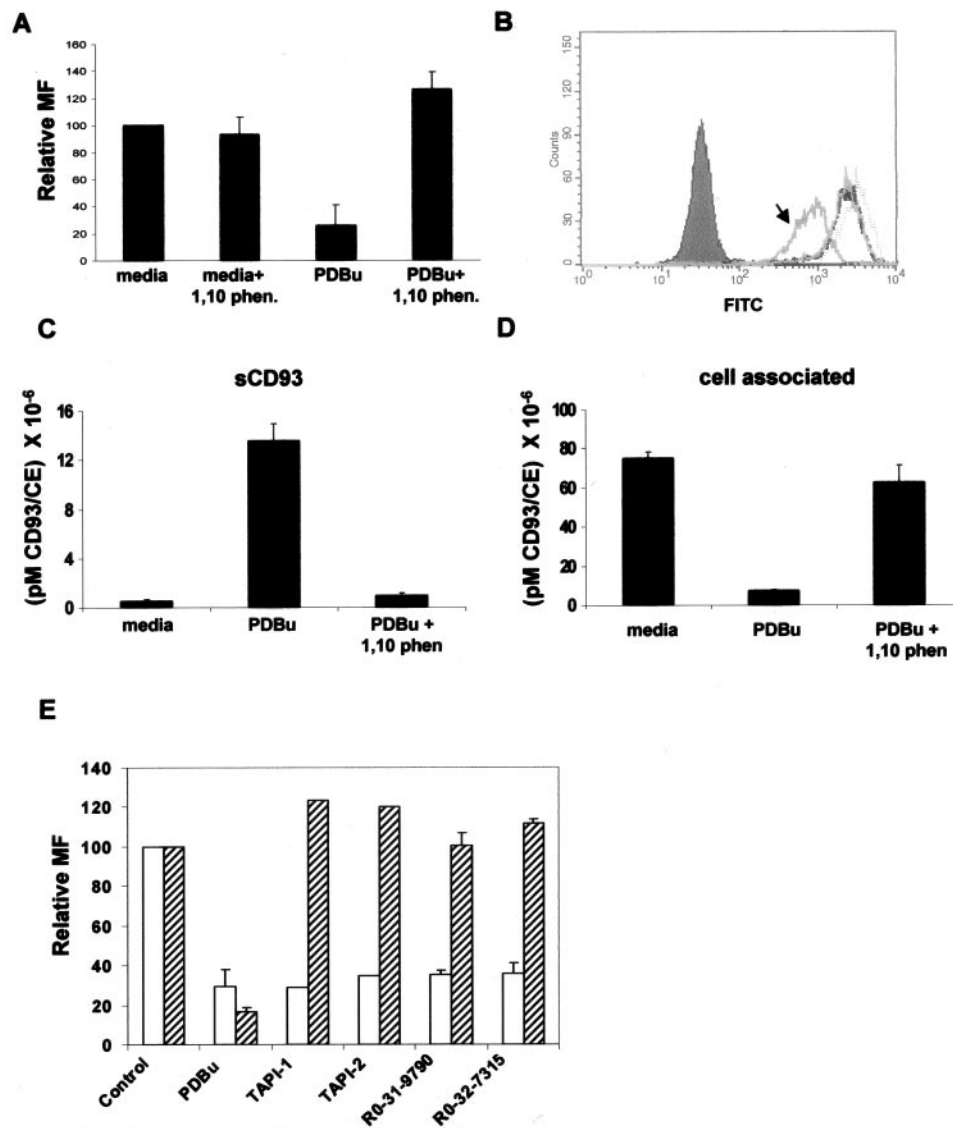


FIGURE 4. CD93 is cleaved from activated monocytes by a metalloproteinase independent of TACE (ADAM17). *A*, Human monocytes (1×10^6) in $500 \mu\text{l}$ were treated with 5 mM 1,10-phenanthroline, stimulated with 50 nM PDBu for 1 h, and analyzed for CD93 expression by flow cytometry. Shown is the average mean fluorescence (\pm SD) relative to the untreated control from two experiments. *B*, Representative histogram from one of the experiments described in *A*. ■, Isotype control. The arrow points to PDBu-stimulated cells. Overlapping histograms are untreated (□) and 1,10-phenanthroline treated (▨). The dotted line represents cells treated with PDBu and 1,10-phenanthroline. *C* and *D*, Monocytes (1×10^7) in $500 \mu\text{l}$ were treated as described in *A*, and supernatants were collected, cells were washed and lysed in $500 \mu\text{l}$ lysis buffer, and $100 \mu\text{l}$ of supernatant (*C*) or $100 \mu\text{l}$ of cell lysate (2×10^6 cell equivalents; *D*) was detected by ELISA. Shown is the average \pm SD from triplicate samples, and data are from one experiment, representative of two. *E*, Monocytes (□) or lymphocytes (▨) were pretreated with TACE inhibitors before addition of 50 nM PDBu. Cells were incubated for an additional 20 min with PDBu, then analyzed for surface CD93 (□) or L-selectin (▨) surface expression by flow cytometry. Treatment with R0 compounds is the average of two experiments, and TAPI compounds were tested in one complete experiment with the L-selectin control (as shown). TAPI compounds showed similar results with CD93 in at least three other experiments (not shown).

and B). Furthermore, sCD93 could not be detected in supernatants from PDBu-stimulated monocytes in the presence of 1,10-phenanthroline (Fig. 4C), and CD93 remained cell associated (Fig. 4D). These data suggest that a metalloproteinase is responsible for the PDBu-induced cleavage event. TACE (ADAM17) is the sheddase responsible for the cleavage of numerous diverse transmembrane molecules (6); therefore, TACE inhibitors were used to determine whether CD93 was cleaved by TACE after PDBu stimulation. Incubation of monocytes with four different hydroxamic acid-based TACE inhibitors, TAPI-1, TAPI-2, R0-31-9790, and R0-32-7315, had no significant effect on PDBu-stimulated shedding of CD93. PDBu-stimulated cleavage of L-selectin on lymphocytes was inhibited by all TACE inhibitors, as expected, verifying that the inhibitors were functional (Fig. 4E).

CD93 is shed from human neutrophils

Because CD93 is also expressed on human neutrophils, albeit at ~5-fold less surface density than monocytes (27) (Fig. 4B vs Fig. 5C), we determined whether similar shedding properties were ev-

ident on this cell type. There was less total cell-associated CD93 detected in PDBu-stimulated human neutrophils compared with untreated control cells, as determined by ELISA (51.2–61.5% range of three experiments; Fig. 5A) and Western blot (Fig. 5D). Furthermore, when cell supernatants were analyzed for the presence of sCD93, 3.75- to 17-fold (range of three experiments) more sCD93 was detected in culture medium from PDBu-stimulated cells compared with unstimulated cells (Fig. 5B), demonstrating that CD93 on neutrophils is also susceptible to PDBu-induced shedding. However, in contrast to monocytes, PDBu-stimulated neutrophils displayed an increase in cell surface CD93 expression (220 ± 69%; $n = 4$; Figs. 5C and 8C). These data indicate that CD93 is shed from PDBu-stimulated neutrophils and also suggests that CD93 may be stored in neutrophil granules, which are released in response to PDBu. In support of this hypothesis, confocal analysis of neutrophils stained with anti-CD93 (1157) demonstrated prominent intracellular staining in permeabilized cells (Fig. 5E, lower right panel), whereas nonpermeabilized cells showed predominantly plasma membrane-associated CD93 (Fig. 5E, upper right).

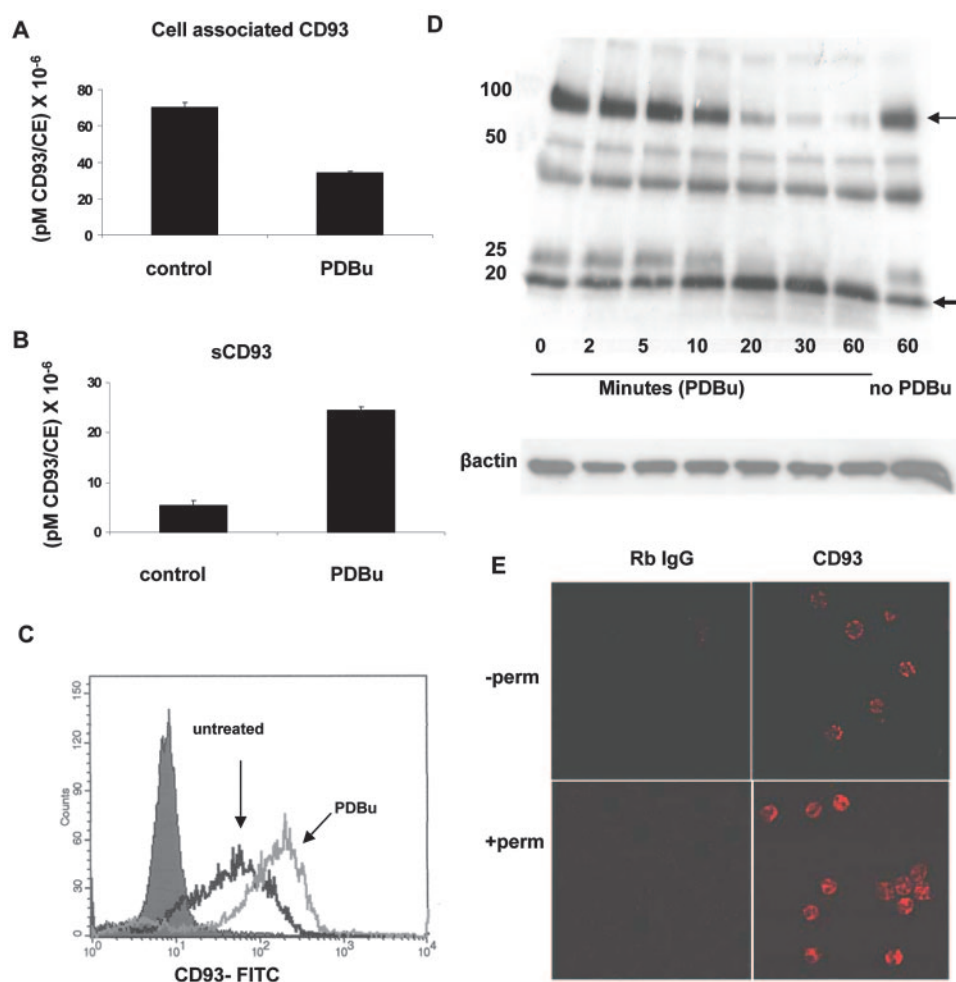


FIGURE 5. CD93 is shed from activated neutrophils, but surface expression is up-regulated. Human neutrophils (1.5×10^7) in 500 μ l were treated with 50 nM PDBu for 15 min, and cell-associated CD93 from 6×10^5 cell equivalents (A) and sCD93 in culture supernatant (not diluted; B) was detected by ELISA. Shown is the average (\pm SD) of triplicate samples from one experiment, representative of three. C, Neutrophil surface CD93 expression after 15-min exposure to PDBu was analyzed by flow cytometry using R3. The histogram is from one experiment, representative of three. ■, IgM control; black line, untreated; gray line, PDBu-treated. D, Equal volumes of cell lysates from human neutrophils treated with 100 nM PDBu for increasing amounts of time were separated by 4–20% gradient SDS-PAGE, transferred to PVDF, and probed with 5 μ g/ml polyclonal anti-CD93 cytoplasmic tail Ab 1150. The blot was stripped and reprobbed for β -actin to control for equal protein loading. The thin arrow points to full-length CD93. The thick arrow points to CD93 cytoplasmic tail containing cell-associated fragment running at <25 kDa. E, Confocal images of nonpermeabilized (upper) and permeabilized (lower) neutrophils labeled with anti-CD93 (right) or control IgG (left).

Glycosylation of CD93 does not alter PDBu-induced cleavage

Previous studies showed that CD93 is heavily *O*-glycosylated, and that hypoglycosylation of CD93 resulted in a loss of surface CD93 (19). Glycosylation of CD93 can be regulated in the CD93-expressing CHO *ldlD* cell line, because growth in medium in the absence of galactose and *N*-acetyl galactosamine (GalNAc) is non-permissive for glycosylation, whereas in the presence of galactose and GalNAc, glycosylation is restored. To determine whether the glycosylation state of CD93 contributed to PDBu-induced shedding, the effect of PDBu on surface CD93 expression of *ldlD*-CD93 cells grown in the presence or the absence of galactose and GalNAc was tested. As shown previously, in the absence of galactose and GalNAc, CD93 surface expression was markedly decreased (average, 60.6% decrease; $n = 3$; data not shown). However, PDBu treatment of cultured glycosylation deficient cells (without sugars) resulted in similar decreases in relative CD93 expression (51.3 and 44.3% in the presence or the absence of sugars, respectively; $n = 3$; Fig. 6) as that in control cells. These data verify that glycosylation of CD93 affects cell surface expression/stability, but the PDBu-induced shedding event is independent of and distinct from the instability induced by hypoglycosylation.

Cross-linking CD93 with immobilized Ab induces shedding

To mimic potential ligation of CD93, monocytes were cultured on anti-CD93 Abs (or isotype control Abs), and cell-associated CD93 was measured by ELISA. After 60 min of adherence to anti CD93, cell-associated CD93 was decreased by $75.0 \pm 13\%$ on R139 compared with IgG2b ($n = 3$), and by 94.1% on R3 compared with IgM ($n = 1$) (Fig. 7A); it remained depressed for at least 18 h in the presence of cross-linking anti-CD93 mAb (decrease of $85.6 \pm 7.6\%$ for R139 and $72.4 \pm 19.5\%$ for R3; $n = 3$; data not shown). The decrease in cell-associated CD93 at 60 min was partially inhibited with 1,10-phenanthroline (19.2 and 29.8% from two separate experiments; data not shown) consistent with ectodomain shedding. Analysis of cell lysates by Western blot demonstrated generation of cell-associated CD93 cytoplasmic tail-containing cleavage products in response to Ab cross-linking (Fig. 7B), verifying that cross-linking stimulated proteolytic cleavage of CD93. However, sCD93 was not detected in supernatants from these adherent cells, even in response to PDBu. This is probably due to the 8- to 10-fold fewer cells per milliliter in the adhesion assay in this study compared with the cell suspension assay (Figs. 1, 4, and 8),

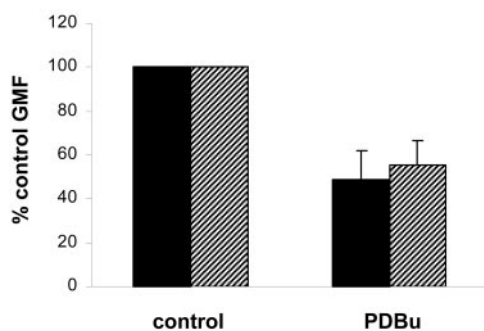


FIGURE 6. PDBu-induced shedding on CHO cells is independent of CD93 glycosylation. CD93-expressing *LdlD* cells were grown in complete medium (with sugars; ■) or in medium lacking galactose and GalNAc (without sugars; ▨). Cells were treated with or without 300 nM PDBu (optimal concentration determined for this cell type) for 30 min and analyzed for surface CD93 expression by flow cytometry. The average geometric mean fluorescence (GMF) relative to control (no PDBu) of three experiments (\pm SD) is shown.

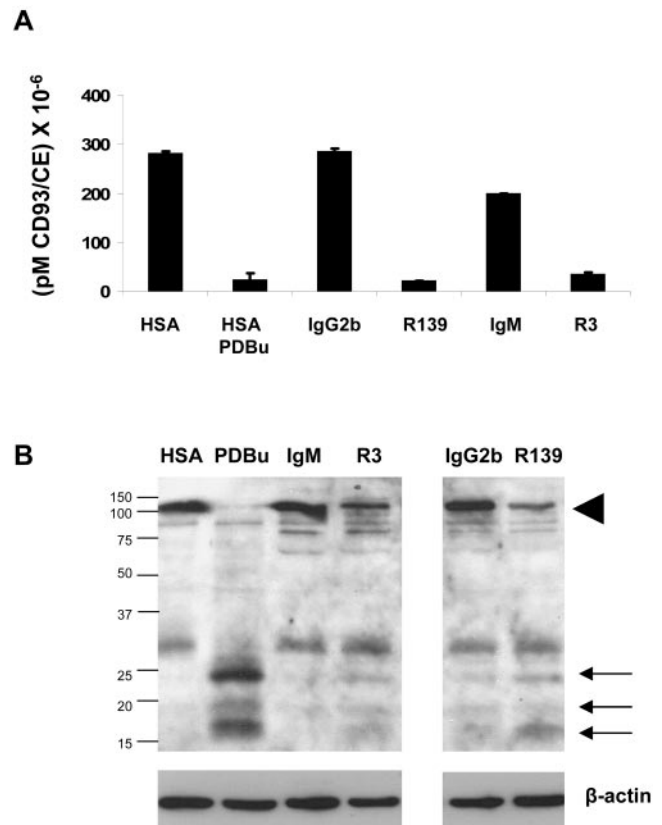


FIGURE 7. Cross-linking CD93 with immobilized Ab triggers shedding of CD93. *A*, Human monocytes (2×10^6) were plated on two-well, Lab-Tek chamber slides coated with 8 μ g/ml HSA or 50 μ g/ml Ab for 1 h at 37°C. After removal of medium and washing, cells were lysed, and lysates were subjected to sandwich ELISA with Abs R3 and R139. Shown is the average of triplicate wells from one experiment, similar to three experiments with R139 and IgG2b. *B*, Cell lysates from *A* were electrophoresed, transferred to PVDF, and probed with an anti-CD93 cytoplasmic tail Ab (1150). Full-length CD93 migrates above the 100-kDa marker (arrowhead), and arrows point to cell-associated proteolytic fragments of CD93 that retain reactivity with the anti-cytoplasmic tail Ab. The blot was stripped and reprobed for β -actin as a loading control.

resulting in sCD93 levels in supernatants that were below the range of sensitivity of the ELISA.

Inflammatory stimuli trigger CD93 shedding from monocytes and alter surface expression on neutrophils

Because the CD93 expression profile (monocytes, neutrophils, and endothelial cells) and its shedding by cell activation are consistent with a role for CD93 in leukocyte extravasation/inflammation, we investigated the influence of inflammatory mediators on CD93 shedding. Human monocytes were treated with the proinflammatory cytokine TNF- α or with the bacterial cell wall component LPS. After 60 min, TNF- α and LPS stimulated shedding of CD93 from monocytes, as assessed by the presence of sCD93 in culture medium (Fig. 8A), although the amount of shed CD93 was 10–25% of that seen with PDBu stimulation. FACS analysis demonstrated that CD93 surface expression after stimulation with TNF- α and LPS was not significantly altered (Fig. 8B), consistent with the lower degree of shed receptor and suggesting a modulated response to alternative stimuli.

In contrast to monocyte CD93 surface expression and consistent with the up-regulation of CD93 expression upon PDBu treatment of neutrophils, TNF- α as well as the inflammatory mediators, C5a

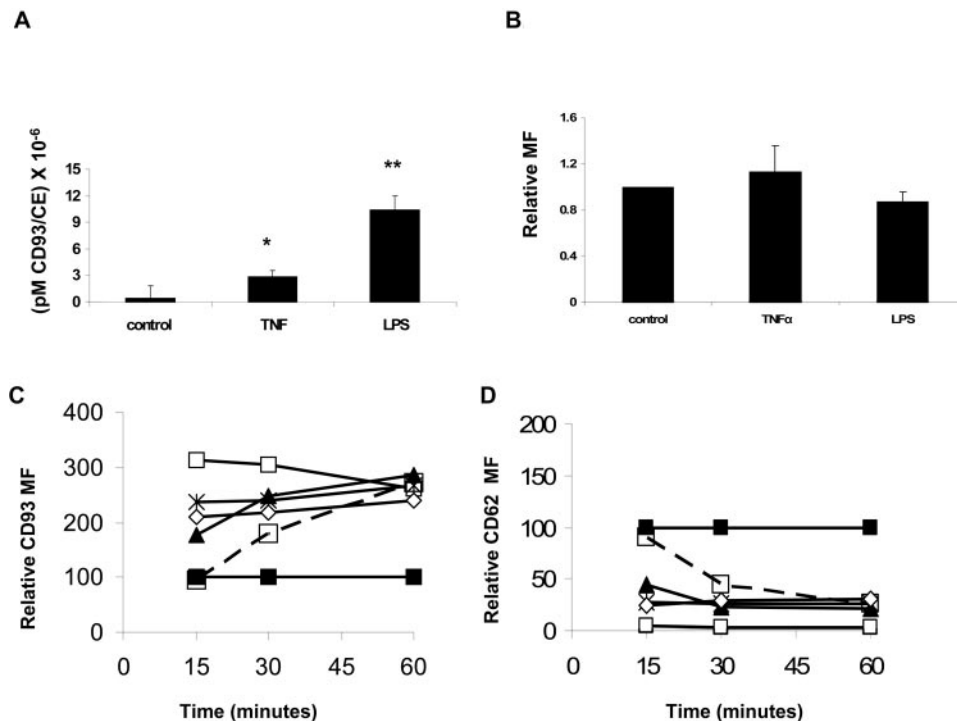


FIGURE 8. Inflammatory mediators induce CD93 shedding on monocytes and alterations in CD93 expression on neutrophils. *A*, Human monocytes (1×10^7) in $500 \mu\text{l}$ of culture medium were treated with or without with 5 ng/ml TNF- α or 100 ng/ml LPS for 60 min at 37°C . Supernatants were analyzed for sCD93 by ELISA. Shown is the mean of triplicate wells (\pm SD) from one experiment representative of two. *, $p = 0.03$; **, $p = 0.003$ (by paired Student's t test). *B*, Surface expression of CD93 from monocytes treated as described in *A* was measured by flow cytometry. Shown is the mean of two separate experiments (\pm SD) relative to the control (untreated) sample. *C* and *D*, Human neutrophils (1×10^6) were incubated at 37°C for various time periods without stimulus (■) or with 5 ng/ml TNF- α (▲), 100 nM C5a (◇), $1 \mu\text{M}$ PAF (X), 50 nM PDBu (□), or 100 ng/ml LPS (□) in HBSS⁺/0.25% HSA and 20 mM HEPES. Surface expression of CD93 (*C*) and L-selectin (*D*) was measured by flow cytometry. A single experiment with multiple time points is shown. Similar results for the 15 min point were obtained in three separate experiments.

and platelet-activating factor (PAF), induced a rapid increase (within 15 min) in CD93 expression on human neutrophils (Fig. 8C). LPS also induced an increase in CD93 expression on neutrophils, but with a slower kinetic (by 30 min). In contrast, these stimuli triggered a decrease in L-selectin expression, suggesting that CD93 and L-selectin may have opposing functions in neutrophil homing (Fig. 8D).

Discussion

This study demonstrates that CD93 on human monocytes and neutrophils is susceptible to protein ectodomain cleavage. The phorbol ester, PDBu, a known inducer of ectodomain cleavage, triggered CD93 cleavage in a time- and dose-dependent manner (Fig. 1). Soluble CD93 was detected in tissue culture supernatants after exposure to PDBu, and two anti-CD93-reactive species were identified by Western blot at 75 and 50 kDa in monocyte supernatants (Fig. 1E). Soluble CD93 was detected in human plasma from healthy donors (Fig. 3), consistent with a physiologic role for sCD93, and the CD93 intracellular domain was detected in cells after shedding. Cleavage of CD93 appears to be mediated by a metalloproteinase, because it was inhibited by the metalloproteinase inhibitor 1,10-phenanthroline (Fig. 4). Cross-linking CD93 with two immobilized mAbs recognizing different extracellular domains of CD93 resulted in the release of CD93 (Fig. 7), suggesting that ligand binding may induce CD93 shedding. However, this decrease in full-length CD93 expression was only partially inhibited by 1,10-phenanthroline, and the ICD-containing cleavage fragments were not as readily detected after Ab cross-linking compared with PDBu stimulation, suggesting that cross-linking may

also stimulate other forms of CD93 degradation insensitive to the metalloproteinase inhibitor. Incubation with the proinflammatory cytokine, TNF- α , or the bacterial cell wall component, LPS, also induced CD93 shedding on monocytes, suggesting that sCD93 may be released at sites of inflammation in vivo (Fig. 8).

Although CD93 was originally described as a C1q receptor that mediated enhanced phagocytosis, recent studies demonstrate that CD93 does not bind to C1q (12) and that CD93 is not required for the C1q-mediated enhanced phagocytic activity (10). McGreal et al. (28) have postulated that CD93, similar to platelet-endothelial cell adhesion molecule 1, is involved in leukocyte homing based on a shared tissue distribution pattern with platelet-endothelial cell adhesion molecule 1 and because a CD93 extracellular domain Fc fusion protein bound to activated endothelium, suggesting that there is a CD93 ligand expressed on the endothelium (12). Therefore, generation of sCD93 induced by proinflammatory mediators (e.g., TNF- α or LPS) or rapid alterations in CD93 expression on activated neutrophils may alter the homing properties of monocytes and/or neutrophils at sites of inflammation. Interestingly, TNF- α , LPS, PAF, and C5a all induced a rapid and nearly complete loss of surface L-selectin expression on neutrophils (Fig. 8D) while inducing an elevated level of CD93 (Fig. 8C), suggesting that these two molecules may have opposing functions in neutrophil migration. Furthermore, the enzyme(s) responsible for cleavage of CD93 differs from the L-selectin sheddase (TACE/ADAM17; Fig. 4D). Shedding independent of TACE/ADAM17 was also reported for CD44 (8), an adhesion molecule with some similarities in structure and function to CD93 (29).

One consequence of ectodomain cleavage is the subsequent regulated cleavage of the transmembrane domain, resulting in the liberation of the ICD into the cytoplasm. Although some ICD fragments are susceptible to proteasome-mediated degradation, others have been shown to act as transcription factors or to act in complex with transcription factors to alter gene expression. Interestingly, the cytoplasmic domain of CD93 contains a highly charged juxtamembrane domain, predicted to contain a nuclear localization signal (30), suggesting that the CD93-ICD may be involved in gene regulation. Furthermore, CD93-ICD fragments are readily detected in human monocyte cell lysates after PDBu treatment or Ab cross-linking (Figs. 1D and 7B), implying a relative lack of susceptibility to proteasome-mediated degradation.

The soluble fragment of the CD93 extracellular domain may have biological activity, because numerous ectodomain cleavage products are biologically active (e.g., TNF- α , TGF- α , TGF- β , and members of the EGF family). Interestingly, Botto et al. (10) showed that CD93-deficient mice were defective in the clearance of apoptotic cells from the inflamed peritoneum; however, the mechanism remains elusive. Perhaps sCD93 is a bridging molecule, responsible for linking the apoptotic cell to the phagocyte, similar to C1q, protein S, thrombospondin, milk fat globule-EGF factor 8 protein, and others (31), and is the component responsible for enhanced clearance of apoptotic cells under inflammatory conditions, when sCD93 would be expected to be present.

The identification of the susceptibility of CD93 to ectodomain cleavage under a variety of conditions and the observation that the soluble fragment of CD93 exists in plasma from normal healthy donors open numerous avenues to study the functional significance of CD93 and the mechanism by which CD93 modulates phagocytosis and adhesion. Although not addressed in this study, CD93 is widely expressed on endothelium, so it will be important to determine whether endothelial CD93 is susceptible to ectodomain cleavage and to characterize the resulting changes in surface expression of CD93 after activation of endothelium. The observations presented in this study warrant further investigation regarding the biological activities of the soluble fragment(s) of CD93 found in plasma, and likely at foci of inflammation, as well as investigations to elucidate the biological activity of the CD93 ICD after ectodomain cleavage.

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Disclosures

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