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CD93 is a cell surface lectin receptor involved in the control of the inflammatory response stimulated by exogenous DNA

Brice Nativel,[1,2] Stéphane Ramin-Mangata,[1,2] Rudy Mevizou,[1] Audrey Figuester,[1] Jessica Andries,[1] Thomas Iwema,[1*] Nobunao Ikewaki,[3] Philippe Gasque[1,4,5‡] and Wildriss Viranaïcken[1,4‡]

[1]*GRI, Groupe de recherche en immunopathologie, EA4517, Université de la Réunion, Saint-Denis, France,* [2]*Université de La Réunion, INSERM 1188, Diabète athérothombose Réunion Océan Indien (DéTROI), Saint-Denis de La Réunion, France,* [3]*Laboratories of Clinical Immunology, Department of Animal Pharmaceutical Science, Welfare School of Pharmaceutical Sciences, Kyushu University of Health, Miyazaki, Japan,* [4]*Université de La Réunion, INSERM 1187, CNRS, 9192, IRD 249, UM 134 Processus Infectieux en Milieu Insulaire Tropical (PIMIT), Saint-Denis de La Réunion, France,* [5]*Laboratoire d'Immunologie Clinique et Expérimentale, ZOI (LICE-OI). CHU site Bellepierre, Saint-Denis de La Réunion, France*

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*Present address:, CALIXAR, 60 Avenue Rockefeller, 69008, Lyon, France
‡Co-senior authors.
Correspondence: Dr Wildriss Viranaïcken and Prof. Philippe Gasque, GRI, Groupe de recherche en immunopathologie, EA4517, Université de la Réunion, Saint-Denis 97400, France.
Emails: wildriss.viranaicken@univ-reunion.fr (W.V.); philippe.gasque@univ-reunion.fr (P.G.)
Senior author: Dr Wildriss Viranaïcken and Prof. Philippe Gasque

Summary

Bacterial DNA contains CpG oligonucleotide (ODN) motifs to trigger innate immune responses through the endosomal receptor Toll-like receptor 9 (TLR9). One of the cell surface receptors to capture and deliver microbial DNA to intracellular TLR9 is the C-type lectin molecule DEC-205 through its N-terminal C-type lectin-like domain (CTLD). CD93 is a cell surface protein and member of the lectin group XIV with a CTLD. We hypothesized that CD93 could interact with CpG motifs, and possibly serve as a novel receptor to deliver bacterial DNA to endosomal TLR9. Using ELISA and tryptophan fluorescence binding studies we observed that the soluble histidine-tagged CD93-CTLD was specifically binding to CpG ODN and bacterial DNA. Moreover, we found that CpG ODN could bind to CD93-expressing IMR32 neuroblastoma cells and induced more robust interleukin-6 secretion when compared with mock-transfected IMR32 control cells. Our data argue for a possible contribution of CD93 to control cell responsiveness to bacterial DNA in a manner reminiscent of DEC-205. We postulate that CD93 may act as a receptor at plasma membrane for DNA or CpG ODN and to grant delivery to endosomal TLR9.

Keywords: bacterial DNA; CD93; CpG oligonucleotide; C-type lectin-like domain; inflammation; Toll-like receptor 9.

Introduction

CD93 is a type 1 transmembrane glycoprotein encoded by the *CD93* gene localized on chromosome 20, position p11.21 in humans.[1,2] CD93 consists of a C-type lectin-like domain (CTLD), five epidermal growth factor-like domains, a mucin domain, a single transmembrane domain and an intracellular domain.[3–5] Expression of CD93 occurs in various cell types including myeloid cells, hematopoietic stem cells, natural killer cells, platelets, neurons, microglia and endothelial cells.[6–8] It was initially shown that CD93 was a receptor for the complement

protein C1q,[9] but this function remains controversial.[10] CD93 regulates several processes involved in innate immunity and inflammation including phagocytosis and adhesion. Previously, it has been shown that CD93 is shed from the surface of activated human monocytes and neutrophils, resulting in the release of a soluble form (sCD93). CD93 shedding was detected under inflammatory conditions.[11] Soluble CD93 induced differentiation of monocytes to macrophage-like cells, as shown by activated cell adhesion and increased phagocytic activities. In addition, this differentiation resulted in an enhanced nuclear factor-κB (NF-κB) response of the THP1 macrophage reporter cell line to several ligands of different Toll-like receptors (TLR) (i.e. TLR1, -2, -5, -6 and -8).[12] Moreover, it was found that sCD93 could boost the tumor necrosis factor-α (TNF-α) secretion of THP1 cells stimulated with lipopolysaccharide (LPS), a TLR4 agonist. The role of sCD93 on the TLR9 signaling pathway is currently unknown.

Several pathogen-associated molecular patterns, alternatively named MAMPs for microbial-associated molecular patterns, are molecules derived from microorganisms which are recognized by cells of the innate immune system. There is a wide variety of MAMPs including bacterial DNA. Bacterial DNA is highly rich in non-methylated CpG oligonucleotides (ODN), which can trigger an innate immune response in different cell types including endothelial cells, monocytes, neutrophils, dendritic cells and macrophages.[12–14] CpG ODN mimic bacterial DNA action. These CpG ODN contribute differentially to cell activation and maturation through their interaction with pattern recognition receptors, such as TLR9 located in endosomes.[15,16] Downstream from this binding, the transcription factor NF-κB is activated and the expression of proinflammatory cytokine [e.g. interleukin-6 (IL-6)] occurs.

Toll-like receptor 9 is present at the cell surface in an inactive form, which acquired the capacity to bind to DNA in the endosomal compartment.[17,18] Due to their negative charges, CpG ODN as well as bacterial DNA need a receptor to bind to the cell membrane, and uptake into endosomes to bind to TLR9. To date, the lectin CD205, also named DEC-205 or Clec13B, has been described as a receptor for CpG ODN on dendritic cells and B cells,[19] allowing delivery to intracellular TLR9 to engage specific signaling pathways. The capacity and the mechanisms through which DEC-205-negative cell types like neurons may respond to bacterial DNA remain ill-characterized. Of note, it has been previously shown that neurons can express CD93 during inflammatory processes such as ischemia encephalitis[20]

DEC-205 also contains a CTLD which is involved in the surface binding and uptake of CpG ODN and bacterial DNA. In light of a common structure organization between DEC-205 and CD93, we hypothesized that the latter could also contribute to cell surface recognition of CpG motifs of bacterial DNA. Interestingly, it has already been shown that CD93 can promote TLR signaling and particularly increase LPS recognition by TLR4 and can boost the inflammatory response of monocytes.[12,21] In analogy, we hypothesized that CD93 may favor the shuttling of exogenous DNA towards TLR9. Using several assays, we herein showed an interaction between either the bacterial DNA or class A CpG ODN with the CTLD of CD93. We found that cell surface CD93 contributes to cell responsiveness to CpG ODN or bacterial DNA in a TLR9-dependent manner.

Material and methods

Reagents

All reagents were from Sigma-Aldrich (St Quentin Fallavier, France), except when indicated. Biotinylated and non-biotinylated CpG-A ODN 2216 (5′-ggGGGACGATCGTCgggggG-3′) and CpG-B ODN 2006 (5′-tcgtcgttttgtcgttttgtcgtt-3′) were from MWG (Eurofins MWG operon, Ebersberg, Germany). Lowercase letters denote a phosphorothioate backbone, and uppercase letters denote a phosphodiester backbone. CpG-A ODN 2216 fluorescein isothiocyanate (FITC) was from InvivoGen (Toulouse, France).

Protein expression and purification

We produced His-human CD93-CTLD in *Escherichia coli* BL21DE3 mainly as described previously.[22] Briefly, the recombinant His-tagged protein was purified using an Ni^{2+} (Ni-NTA) column followed by gel-filtration. During the test experiments, we found that we had obtained, by gel-filtration, high-molecular-weight complexes corresponding to bacterial DNA associated with CTLD domains despite digestion with DNAse I at 80 U/ml for 30 min at 25° after the affinity step. We therefore followed the protocol previously developed in the laboratory[22] with minor modifications. During affinity chromatography, we performed successive washes each time with 20 column volumes of 25 mM HEPES, 1·5 M NaCl, then with 25 mM HEPES, 20 mM imidazole, then with 25 mM HEPES, 0·1% (volume/volume; v/v) Triton X-114, 20 mM imidazole and then a final washing step with 25 mM HEPES, 20 mM imidazole. Remaining bound DNA was removed using 1·5 M NaCl instead of 0·5 M. The LPS was eluted from the column with the use of 0·1% (v/v) Triton X-114. Gel-filtration was performed on S-200 Sephadex in 25 mM HEPES.

GelRed agarose gel electrophoresis was carried out to confirm that the recombinant protein preparation lacks DNA or RNA. *Limulus* amebocyte lysate assay was carried out to verify that the recombinant protein preparations lacked contamination by LPS. The level of LPS was under 1 ng/l. The proteins were quantified by bicinchoninic acid assay. All recombinant proteins were stored at −80° after being flash-frozen in liquid nitrogen.

SDS–PAGE and Western blotting analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were conducted as previously described.[22] For Western blotting, the membrane was incubated with a monoclonal antibody against human CD93 (clone mNI-11, obtained from Dr Ikewaki) at 1 : 1000 dilution. Membranes were washed three times for 5 min and incubated with a 1 : 2000 dilution of horseradish peroxidase (HRP) -conjugated goat anti-mouse antibody (Vector Labs, CliniSciences, Nanterre, France) for 1 hr. Membranes were washed with TRIS buffered saline (TBS) –Tween-20 (0·05%) three times and developed with the Novared system (Vector Labs) according to the manufacturer's protocol.

Bacterial DNA preparation

Escherichia coli DNA was extracted from BL21(DE3) Clear Coli as before.[23] Briefly, bacteria from an overnight culture in 1 liter of Luria Bertani broth were pelleted and resuspended in 8 ml of 25 mM HEPES pH 7.2. Then cells were lyzed by sonication. After heating at 95° for 10 min, denatured extract was centrifuged at 20 000 *g*, 25° for 20 min. Supernatants were mixed with an equal volume of EDTA saturated phenol. Aqueous phase was recovered by centrifugation as before and then mixed with an equal volume of chloroform. Aqueous phase was recovered as above and precipitated with 0·3 M sodium acetate pH 5.2 and 1.5 volume of absolute ethanol followed by an incubation overnight at −20°. DNA was pellet at 12 000 *g*, 4° for 15 min. After a washing step with 80% ethanol, DNA was resuspended in 25 mM HEPES pH 7·2. DNA was quantified at 260 nm and a 260 : 280 ratio was consider acceptable when it was >1·8.

ELISA to test for CTLD–DNA interactions

Maxisorp microplates (Nunc, Roskilde, Sjaelland, Denmark) were coated overnight at 4° with either 100 µl of *E. coli* bacterial DNA at 10 µg/ml or CpG ODN at 1 µM in phosphate-buffered saline (PBS). Wells were washed with TBS (pH 7·5) once and with TBS-Tween 0.1% (v/v) three times followed by saturation with bovine serum albumin (BSA) 5% in TBS-Tween. The plates were then incubated for 30 min and washed three times as previously. After washing, plates were overlaid with CD93-CTLD at a concentration between 0 and 10 µg/ml in TBS with Tween and BSA 5% for 2 hr. Plates were then washed. The mouse anti-CD93 (clone P1C5, in-house) (1 : 500) was used as a primary antibody for 1 hr. An HRP-conjugated goat anti-mouse was used next as secondary antibody. TMB One (Promega, Charbonnières-les-Bains, France) was used for the revelation and reaction was stopped with HCl 0·2 M. Absorbance was read at 450 nm with a reference filter at 570 nm.

Alternatively, the Maxisorp microplate was coated with 100 µl CD93-CTLD at 1 µg/ml and saturated as above. Biotinylated CpG was added at different concentrations ranging from 0 to 10 µM. The plate was incubated 30 min at room temperature, then rinsed and HRP-conjugated streptavidin was added at 1/1000 (Vector Labs). After washing, ELISA was revealed as above. For electrostatic experiments, during the capture of CpG by CD93-CTLD, the pH was 8·5 or 6·5 instead of 7·5. After this step, protein–DNA complexes were cross-linked by glutaraldehyde at 4% during 10 min followed by three washing steps with TBS-Tween at pH 7·5. Detection was performed as above.

Fluorescence experiments

Intrinsic tryptophan fluorescence studies of CD93-CTLD were performed as described previously.[22] Briefly, we used 150 µg/ml of CD93-CTLD diluted in PBS at 30°. The interaction of CD93-CTLD with *E. coli* bacterial DNA or CpG ODN, was investigated by recording the fluorescence emission spectra of the CD93-CTLD exposed to increasing concentrations of *E. coli* DNA. A tryptophan fluorescence spectrum was collected using the Horiba spectrofluorimeter (Fluoromax4) with an excitation wavelength at 295 nm or 495 nm with a slit of 5 nm. Emission spectra were measured in the wavelength range of 320–360 nm.

Cell culture and cell transfection

IMR32 cells were transfected with PDR2 empty vector (PDR2-EV) or PDR2-CD93, as described previously.[22] IMR32 and HEK293 transfected cells were cultured in RPMI-1640 completed with 10% (v/v) fetal bovine serum, 2 mmol/ml glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5 µg/ml amphotericin B (PAN Biotech, Aidenbach, Germany) at 37°, 5% CO_2 in a humidified cell culture incubator.

HEK293 null-blue and HEK293 TLR9-Blue (InvivoGen) cells were transfected with plasmid PDR2-EV or PDR2-CD93 using lipofectamine 3000, as recommended by the supplier (Thermo Fisher, Illkirch-Graffenstaden, France). The cells were treated 20 hr later with 10 µM CpG-A for 16 hr. The activation of NF-κB/AP1 was then measured using a quanti-blue assay on culture supernatants according to the supplier's instructions (InvivoGen).

Flow cytometry

IMR32 cells transfected with PDR2-EV or PDR2-CD93 (full length) were harvested after two washes with PBS, then pelleted at 800 *g* for 5 min. Cells were incubated with 5% (weight/v) BSA in PBS for 20 min at room temperature under agitation. To test for the expression of CD93 at the cell surface, primary antibody was anti-CD93

(mNI-11, 1 mg/ml) or isotype control [mouse IgG1a ctrl (X0931; Dako, Santa Clara, CA), 10 mg/ml] at 1 : 100 dilution and secondary antibody was anti-mouse conjugated to R-phycoerythrin (polyclonal antibody; Southern Biotechnology, Birmingham, AL) at 1 : 500 dilution. After two washes with PBS, cells were harvested in PBS. For each experiment 10 000 cells were analyzed by flow cytometry (Cytoflex; Beckman coulter, Brea, CA; CytExpert 2.1 software).

For the CpG-A binding study, cells were incubated with CpG-A coupled to FITC (InvivoGen) at 10 μM. After two washes with PBS, cells were harvested in PBS. For each experiment 10 000 cells were analyzed by flow cytometry (Beckman Coulter FC-500-MPL Flow Cytometer, MXP acquisition software and CXP analysis software, version 2.2).

Interleukin-6 and TNF-α cytokine quantification

Cells were plated in 96-well plates at a density of 1×10^5 cells/ml. For treatment, cells were treated with *E. coli* DNA (10 μg/ml) or CpG-A (10 μM) for 20 hr. Supernatants were collected and the amount of cytokine was measured by ELISA (Ready-SET-Go!®; eBioscience, Vienna, Austria) according to the manufacturer's protocols.

Statistical analysis

All data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 6 Software, Inc. (La Jolla, CA) Shapiro–Wilk normality tests were performed with an α value of 0·05 before using one-way analysis of variance, followed by Tukey's test to compare each treatment. P values <0·05 were considered significant.

Results

CD93-CTLD interacts with bacterial DNA

It is well established that a great diversity of lectins with a CTLD play an important role to selectively recognize pathogens such as bacterial DNA[24] and that the CTLD domain alone is sufficient for DNA binding. Hence, we decided to focus on the CTLD domain of CD93 during this study. Given that interaction studies require a significant amount of recombinant proteins, we decided to produce and purify the CTLD domain in a bacterial expression system (*E. coli*) as before.[22]

To test further the hypothesis that CD93-CTLD could interact with bacterial DNA, we first performed an ELISA using *E. coli* DNA and highly purified recombinant human CD93-CTLD protein (see Supplementary material, Fig. S1a). We observed that CTLD-CD93 was able to bind to immobilized *E. coli* DNA in a dose-dependent manner (Fig. 1a).

Another assay was used to validate the protein–DNA interactions in an aqueous environment and by measuring the fluorescence of intrinsic tryptophan of CD93-CTLD (tryptophan fluorescence assay). We found that CD93-CTLD tryptophan fluorescence decreased when incubated with *E. coli* DNA. The response was dose-dependent and may be related to an interaction between the two molecules (Fig. 1b).

CD93-CTLD interacts with CpG ODN

CpG was used to mimic pathogen-derived DNA and to test the hypothesis that CD93-CTLD could interact with CpG motifs in bacterial DNA. We performed an ELISA using CpG-A or GpC-A as overlay molecules while His-CD93-CTLD was immobilized on the plate. We observed a dose-dependent interaction between CpG-A and GpC-A and CD93-CTLD (Fig. 2a). CpG-A binding to CD93-CTLD was more pronounced when compared with that of GpC-A. Of note, we further tested if immobilized CpG were able to interact with overlaid CD93-CTLD (data not shown). We found that CD93-CTLD was able to bind to immobilized CpG motifs.

We conducted spectrofluorescence analysis of CD93-CTLD in the presence or not of CpG-A as before mentioned. Remarkably, CD93-CTLD tryptophan fluorescence decreased in a dose-dependent manner with increasing doses of CpG-A, indicating an interaction between CpG-A and CD93-CTLD (Fig. 2b). We performed a gel shift assay and confirmed the binding of CTLD-CD93 to CpG-A (see Supplementary material, Fig. S1b).

To show that the observed interaction between the ODN CpG and the CTLD domain is dependent on a correct folding of the domain, we repeated the ELISA binding assay experiment in the presence of the mNI-11 anti-CD93 antibody. This is a unique antibody against the CTLD domain of CD93 and its monovalent form (Fab) has been shown to induce homotypic aggregation of CD93-expressing U937 cells stimulated with LPS.[25] Interestingly, this antibody was able to neutralize the binding of CpG to the CTLD of CD93 (see Supplementary material, Fig. S2).

CD93-CTLD interacts with DNA in a sequence-independent manner

Given that CpG-A and GpC-A were able to interact with CD93-CTLD, we tested if the binding to DNA is sequence-independent and/or conformation-independent. Using the tryptophan fluorescence assay, single-stranded DNA (R-HMGB1 and F-HMGB1), as well as double-stranded DNA [ds-HMGB1 or polyd(IC)], were able to interact with CD93-CTLD. The interaction was also

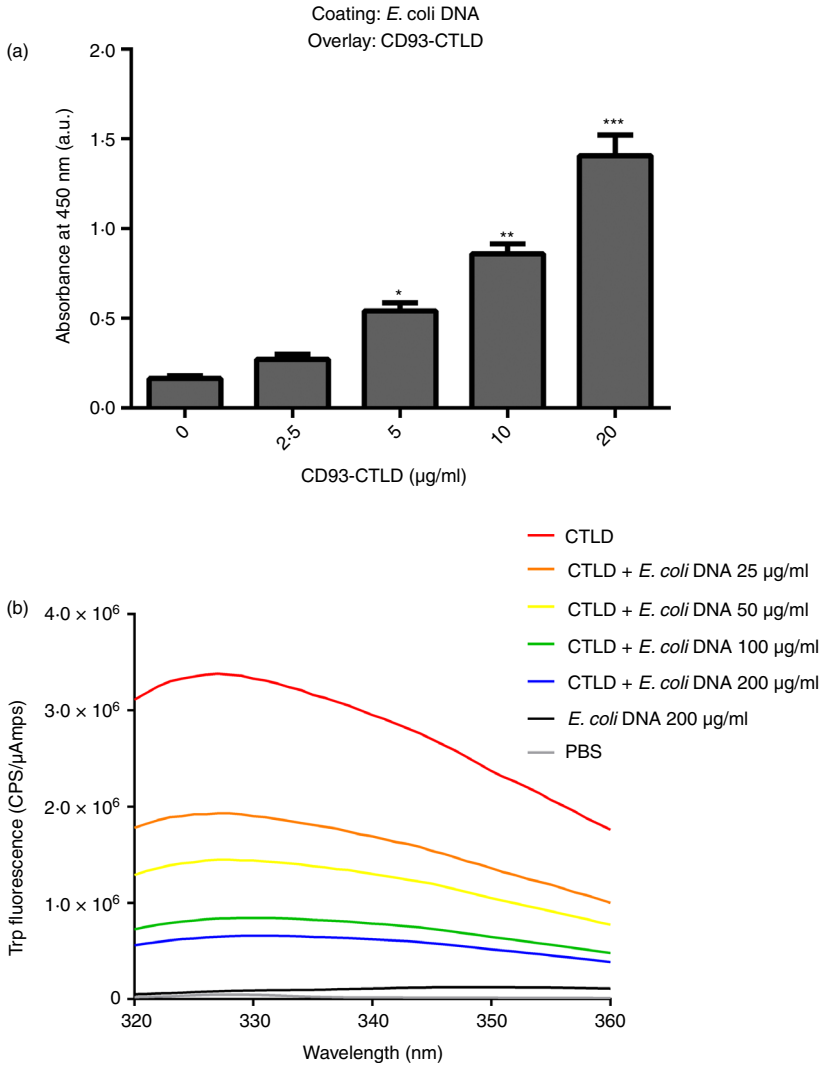

Figure 1. Bacterial DNA can bind to soluble CD93-C-type lectin-like domain (CTLD). (a) Microplate was coated with *Escherichia coli* DNA at 10 µg/ml. After washing and saturation, plates were overlaid with His-CD93-CTLD between 0 and 10 µg/ml. Results are presented as the mean ± SEM from three independent experiments. Differences were determined by one-way analysis of variance Tukey's test: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ compared with control. (b) Analysis of bacterial DNA binding to His-CD93-CTLD by spectrofluorescence. His-CD93-CTLD (at 150 µg/ml) was incubated with *E. coli* DNA ranging from 0 to 200 µg/ml. Interactions were monitored by the quantification of tryptophan fluorescence with an excitation at 295 nm and emission at 330 nm. Results are representative of three independent experiments.

reported between CD93-CTLD and native as well as denatured CpG (Fig. 3a). Taken together these observations indicate a probable interaction in a sequence-independent manner between DNA and CD93-CTLD, possibly through binding to the sugar–phosphate backbone.

DNA has negative charges through the phosphate groups. We hypothesized that the interaction between DNA and CD93-CTLD may involve electrostatic charges with the CTLD being positively charged. The theoretical isoelectric point of the recombinant CD93-CTLD is 8·97 (ExPAXy, compute pI/Mw). We performed the CD93-DNA ELISA at different pH. Interestingly, CD93-CTLD binding was at its maximum at pH 6·5 and 7·5 and was dramatically reduced at pH 8·5, which is close to the isoelectric point of the molecule (Fig. 3b). Hence, we could conclude that DNA binding to CD93-CTLD involved the positive charges of the protein.

CpG ODN binds to IMR32 neuroblastoma expressing cell surface CD93

All the above experiments were performed using recombinant CD93-CTLD produced in bacteria and which lack post-translational modifications. The next step was to over-express the full-length CD93 protein in the IMR32 neuroblastoma cell line lacking endogenous expression of CD93. This neuroblastoma cell line has been shown to express TLR9 at the mRNA level by RT-PCR and at the protein level by Western blot and flow cytometry.[26] We confirmed that our IMR32 cell line clone expressed constitutively the endosomal DNA receptor TLR9 (see Supplementary material, Fig. S3a). The IMR32 human neuroblastoma cell line was either transfected with CD93 plasmid (PDR2-CD93) or empty plasmid (PDR2-EV). Of further critical note, we found that the expression of TLR9 was not significantly altered when IMR32 cells were transfected for CD93 (see Supplementary material,

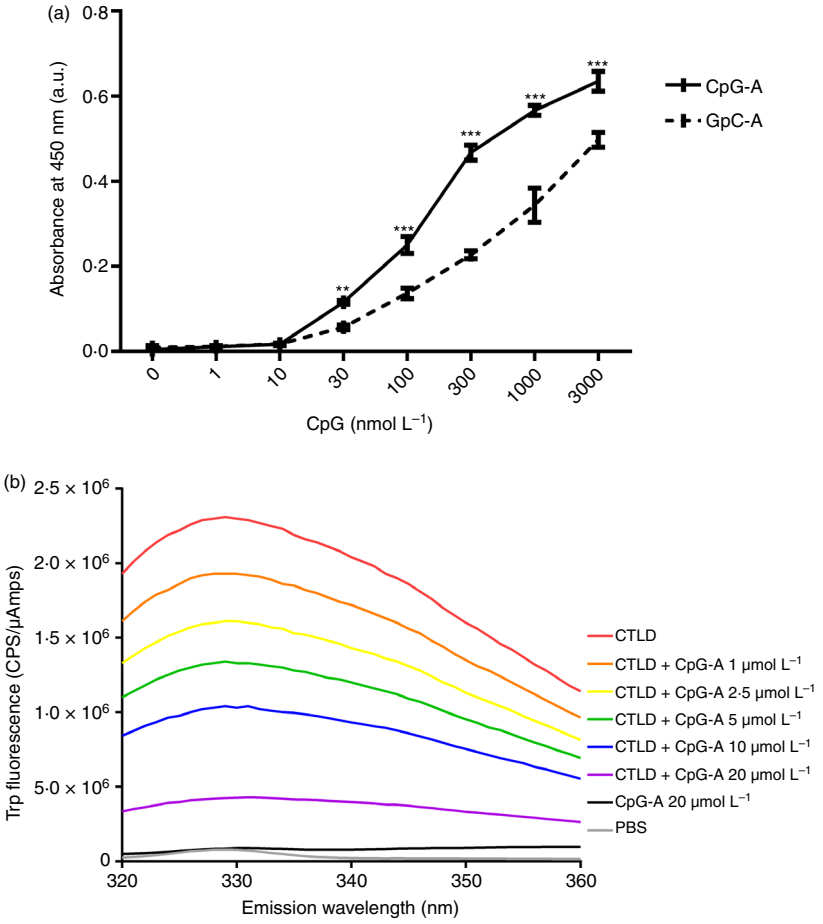

Figure 2. Immunostimulatory CpG-A interacts with the C-type lectin-like domain (CTLD) of CD93. (a) Microplate was coated with His-CD93-CTLD at 1 µg/ml. After washing and saturation, plates were overlaid with biotinylated CpG-A or GpC-A ranging from 0 to 3 µM. Streptavidin–horseradish peroxidase was used for the revelation. Results presented are representative of three independent experiments. Differences were determined by one-way analysis of variance Tukey's test: $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ compared with control. (b). Analysis of CpG-A binding to His-CD93-CTLD by spectrofluorescence. His-CD93-CTLD (at 150 µg/ml) was incubated with CpG-A at different concentrations (0–20 µM). Interactions were monitored by the quantification of tryptophan fluorescence with an excitation at 295 nm and emission at 330 nm. Results are representative of three independent experiments.

Fig. S3a). CD93 cell surface expression was tested by cytometry analysis using the monoclonal antibody mNI-11 raised against the CTLD domain (see Supplementary material, Fig. S3b). Untransfected IMR32 cells did not show cell surface expression of CD93 when compared with high levels in CD93-transfected IMR32 cells.

Using CpG-A FITC, only low level binding was observed on IMR32 cells transfected with PDR2-EV (Fig. 4a). In contrast, more robust binding of the CpG-A was observed on CD93-transfected MR32 cells (Fig. 4a).

CpG ODN and bacterial DNA stimulated IL-6 secretion by CD93-IMR32 transfected cells

Having established that CD93 and DNA can interact at the cell surface, we wanted to ascertain the possible functional consequences of this interaction. To analyze the capacity of CD93 to modulate extracellular DNA responses, we used IMR32 cells transfected or not for CD93. Cells were stimulated with either *E. coli* DNA or CpG-A and analyzed for the IL-6 secretion by ELISA. We found that *E. coli* DNA as well as CpG-A induced a more robust secretion of IL-6 in cells expressing CD93 when compared with control cells (Fig. 4b).

To support these observations using the neuroblastoma model, we tested the contribution of CD93 in the CpG response using the monocyte cell line U937, which is known to express constitutively CD93 and TLR9. These expressions were also validated herein by RT-PCR (see Supplementary material, Fig. S5a). Next, we used mNI-11 to block the interaction between CD93 and CpG-A (see Supplementary material, Fig. S5b) and tested TNF-α secretion by ELISA. We found that the TNF-α secretion induced by the addition of CpG-A was significantly decreased and not for addition of lipotenic acid in the presence of the blocking anti-CD93 CTLD antibody.

CD93 contributes to NF-κB activation in a TLR9-dependent manner following exposure to CpG ODN

We were able to show that CD93 binds to extracellular DNA and modulates IL-6 or TNF-α secretion in two different model cell lines, IMR32 neuroblastoma or U937, respectively. These two cell types express the TLR9 receptor (see Supplementary material, Figs S3a and S5a). The next set of experiments was designed to specifically addressed whether CD93-mediated activation was dependent on TLR9 and to identify the signaling pathway

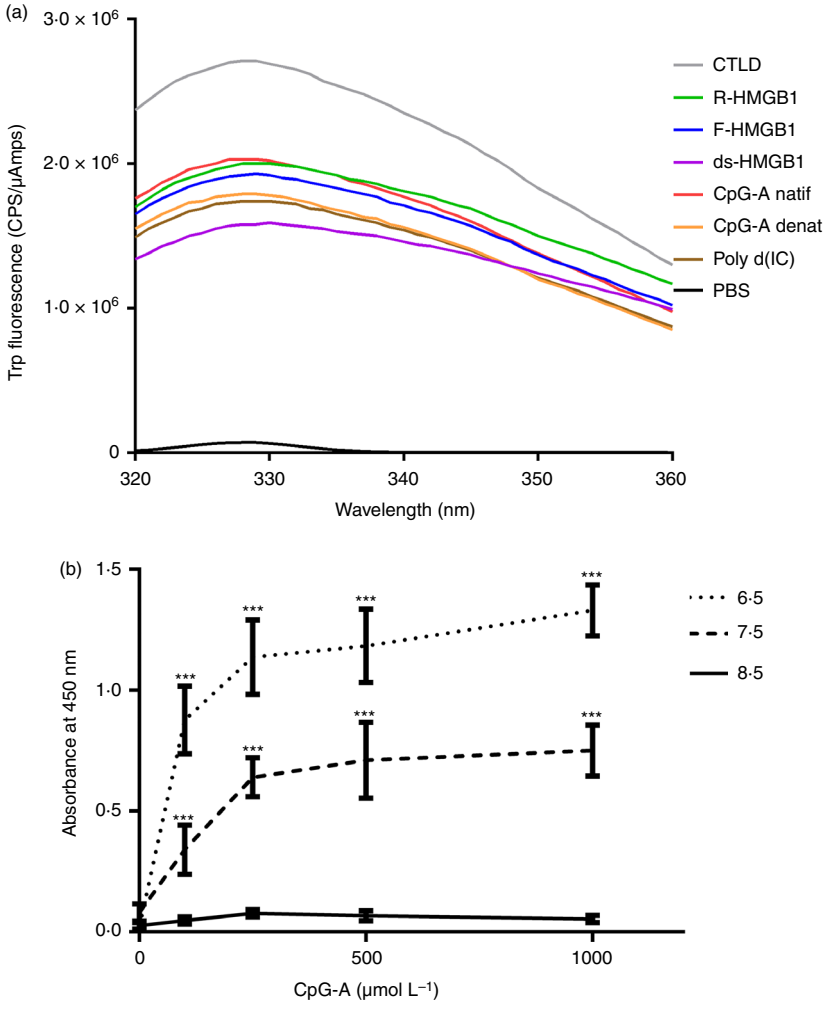

Figure 3. CD93- C-type lectin-like domain (CLTD) interacts with DNA independently of DNA sequences and structure in a pH-dependent manner. (a) Analysis of different DNA to His-CD93-CTLD by spectrofluorescence. His-CD93-CTLD (at 150 µg/ml) was incubated with different DNA. Interactions were monitored by the quantification of tryptophan fluorescence with an excitation at 295 nm and emission at 330 nm. Results are representative of three independent experiments. DNA used were single-stranded DNA (R-HMGB1 and F-HMGB1) double-stranded DNA [ds-HMGB1 and Poly d(IC)], native CpG-A and denatured CpG-A. Results are representative of three independent experiments. (b) Microplate was coated with His-CD93-CTLD at 1 µg/ml. After washing and saturation, plates were overlaid with biotinylated CpG-A at 1 µM at different pH (6·5, 7·5 and 8·5) after a cross-linking and washing step, streptavidin–horseradish peroxidase was used for the revelation. Differences were determined by one-way analysis of variance Tukey's test: ***$P < 0{\cdot}001$ compared with control.

engaged downstream of the CD93–TLR9 complex interacting with DNA. We first transfected 293 null blue cells and 293 blue TLR9 cells with the CD93 full lenght coding plasmid. Cells were also expressing a reporter gene to screen for the activation of NF-κB-AP1 factor (blue system). We treated the cells with CpG-A and monitored the NFκB-AP1 activation by measuring the alkaline phosphatase activity in the supernatant. We used control cells or stimulated with CpG-A for 16 hr. CD93 expression does not induce a change in TLR9 expression in this model (see Supplementary material, Fig. S6).

As shown in Fig. 5(a), we found that the CpG-A response (increased levels of alkaline phosphatase activity) was significantly more important in CD93-transfected cells when compared with mock-transfected cells and this effect was dependent on TLR9 expression (Fig. 5).

Discussion and conclusion

The innate immune activation in response to either pathogens (pathogen-associated molecular patterns, e.g. DNA virus, bacteria) or tissue damage (damage-associated molecular patterns or alarmins, e.g. mitochondrial DNA) can contribute to exacerbate central nervous system inflammation. Damaged cells in the central nervous system may also release exosomes containing fragmented cellular DNA from dying cells, which can stimulate endosomal TLRs such as TLR9. In contrast to the well-described response of glia or infiltrating immune cells where TLR signaling induces cytokine production and/or cell proliferation, the effects of TLR stimulation of neurons by exogenous DNA remain largely ill-characterized.[27]

However, neurons have been reported to express all endosomally located TLRs (TLR3, TLR7, TLR8 and TLR9), which may be implicated in mechanisms of cell death but through ill-characterized mechanisms.[28]

Toll-like receptor 9 is a canonical receptor that engages the innate immune response mediated by pathogenic DNA released from microbes and also from necrotic cells, where cell-derived DNA will act as major alarmins. TLR9 signaling can have contrasting effects: mediating either protective or pro-apoptotic activities. Cell surface TLR9 cannot bind to DNA but will acquire this capacity while localized in the endosomal compartment.[17,18] As a result

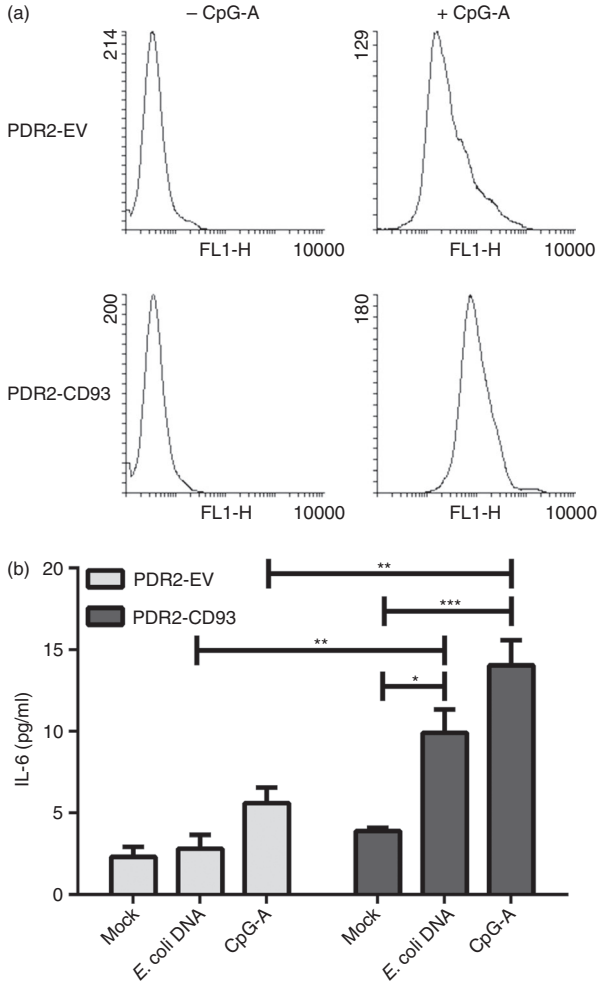

Figure 4. CpG and bacterial DNA boost interleukin-6 (IL-6) expression in CD93-transfected cells. (a) IMR32 (CD93-negative cells) cells were transfected with or without CD93 (PDR2-CD93 and PDR2-EV, respectively). Transfected IMR32 cells were pelleted then were incubated with or without CpG-A coupled to FITC (10 μM). Cells were analyzed by flow cytometry. Results are representative of three independent experiments. (b) Transfected IMR32 cells (5×10^5 cells/ml) were treated with either *Escherichia coli* DNA at 10 μg/ml or CpG-A at 10 μM. The secretion of IL-6 in the supernatant was evaluated by ELISA. Results are presented as the mean ± SEM from three independent experiments. Differences were determined by one-way analysis of variance Tukey's test: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

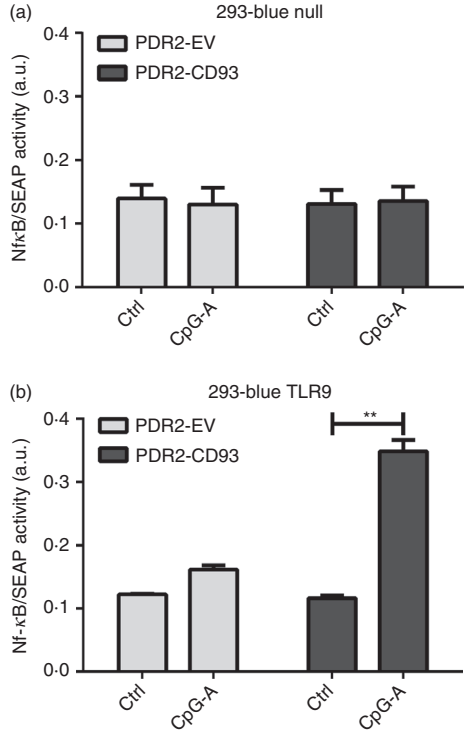

Figure 5. CD93 contributes to nuclear factor-κB (NF-κB) activation in a Toll-like receptor 9 (TLR9) –dependent manner. 293 blue cells (a) or 293 Blue TLR9 cells (b) transfected with pDR2-EV or PDR2-CD93 were treated with CpG-A at 10 μm for 16 hr. The secretion of alkaline phosphatase in the supernatant was evaluated using a Quanti-blue assay. Results are presented as the mean ± SEM from three independent experiments. Differences were determined by one-way analysis of variance Tukey's test: $**P < 0{\cdot}01$.

of their negative charges, CpG ODN as well as bacterial DNA need a receptor to bind to cell membrane and uptake into endosomes to bind to TLR9.

We show for the first time that CD93 interacts with immunostimulatory DNA through its CTLD domain. This interaction involved electrostatic interactions and phosphate–sugar moieties (Figs 1, 2 and 3). Our results are in agreement with the capacity of CpG motifs in bacterial DNA to interact with the CTLD of CD93. Moreover, we demonstrated that CD93 interaction with CpG motifs in bacterial DNA could modulate the proinflammatory activity of the IMR32 neuron cell line transfected to express CD93 (Fig. 4). Using reporter HEK293 cells we were able to show that the proinflammatory response is mediated through the TLR9 pathway and activates the transcription factor NF-κB (Fig. 5). If the role of TLR9 on neurons may be critical to respond to DNA released, for instance, by dying brain cells, we cannot exclude that the cGAS-mediated cytosolic DNA sensing may also be engaged.[29]

DEC-205 is an important lectin and co-receptor to deliver DNA to endosomal TLR9 but its expression is mainly restricted to myeloid-derived immune cells. To our knowledge, the expression of this lectin by neurons has never been reported. In contrast, we have previously described that CD93 is expressed by neurons and particularly in disease conditions.[7]

Interestingly CD93 knockout mice have been shown to have increased severity in the context of experimentally induced ischemia and encephalitis.[20,30] The brain tissue damage created in these models is likely to contribute to a major release of CpG-rich DNA from, for example, mitochondria from injured cells. We believe that the lack

of CD93 on neurons in the knockout animals may result in an impaired response to DNA and failure to engage salutary mechanisms of cell survival mediated through NF-κB.

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Disclosures

The authors declare that they have no competing interests.

Author contributions

WV, TI and PG designed the research; BN, SRM, RM, AF, JA, TI, WV and PG performed the research; BN, SRM, RM, AF, JA, TI, WV., NI. and PG contributed new reagents/analytic tools; all authors analyzed the data; and BN, PG and WV wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Purification and interaction of CD93-C-type lectin-like domain.

Figure S2. mNI-11 antibody antagonizes interaction of CD93-C-type lectin-like domain with CpG-A.

Figure S3. IMR32 and IMR32-CD93 express comparable levels of Toll-like receptor 9 mRNA.

Figure S4. IMR 32 and IMR32-CD93 do not express CD205 at mRNA level.

Figure S5. mNI-11 antibody blocks transactivation of U937 cells mediated by CpG-A but not by lipotenic acid.

Figure S6. CD93 expression does not change Toll-like receptor 9 expression on 293 reporter cells.