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ORIGINAL ARTICLE ***Andrology***

Fucosylated clusterin in semen promotes the uptake of stress-damaged proteins by dendritic cells via DC-SIGN

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STUDY QUESTION: Could seminal plasma clusterin play a role in the uptake of stress-damaged proteins by dendritic cells?

SUMMARY ANSWER: Seminal plasma clusterin, but not serum clusterin, promotes the uptake of stress-damaged proteins by dendritic cells via DC-SIGN.

WHAT IS KNOWN ALREADY: Clusterin is one of the major extracellular chaperones. It interacts with a variety of stressed proteins to prevent their aggregation, guiding them for receptor-mediated endocytosis and intracellular degradation. The concentration of clusterin in semen is almost 20-fold higher than that found in serum, raising the question about the role of seminal plasma clusterin in reproduction. No previous studies have analyzed whether seminal plasma clusterin has chaperone activity. We have previously shown that seminal plasma clusterin, but not serum clusterin, expresses an extreme abundance of fucosylated glycans. These motifs enable seminal plasma clusterin to bind DC-SIGN with very high affinity.

STUDY DESIGN, SIZE, DURATION: *In vitro* experiments were performed to evaluate the ability of seminal plasma clusterin to inhibit the precipitation of stressed proteins, promoting their uptake by dendritic cells via DC-SIGN (a C-type lectin receptor selectively expressed on dendritic cells (DC)). Moreover, the ability of seminal plasma clusterin to modulate the phenotype and function of DCs was also assessed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Clusterin was purified from human semen and human serum. Catalase, bovine serum albumin, glutathione S-transferase, and normal human serum were stressed and the ability of seminal plasma clusterin to prevent the precipitation of these proteins, guiding them to DC-SIGN expressed by DCs, was evaluated using a fluorescence-activated cell sorter (FACS). Endocytosis of stressed proteins was analyzed by confocal microscopy and the ability of seminal plasma clusterin-treated DCs to stimulate the proliferation of CD25+FOXP3+CD4+ T cells was also evaluated by FACS.

MAIN RESULTS AND THE ROLE OF CHANCE: Seminal plasma clusterin interacts with stressed proteins, inhibits their aggregation ($P < 0.01$) and efficiently targets them to dendritic cells via DC-SIGN ($P < 0.01$). DCs efficiently endocytosed clusterin-client complexes and sorted them to degradative compartments involved in antigen processing and presentation. Moreover, we also found that the interaction of seminal plasma clusterin with DC-SIGN did not change the phenotype of DCs, but stimulates their ability to induce the expansion of CD25+FOXP3+CD4+ T lymphocytes ($P < 0.05$ versus control).

LIMITATIONS, REASONS FOR CAUTION: All the experiments were performed *in vitro*; hence the relevance of our observations should be validated *in vivo*.

WIDER IMPLICATIONS OF THE FINDINGS: Our results suggest that by inducing the endocytosis of stress-damaged proteins by DCs via DC-SIGN, seminal plasma clusterin might promote a tolerogenic response to male antigens, thereby contributing to female tolerance to seminal antigens.

STUDY FUNDING/COMPETING INTEREST(S): The present research was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas, the Buenos Aires University School of Medicine, and the Agencia Nacional de Promoción Científica y Tecnológica (Argentina). The authors have no conflicts of interest to declare.

Key words: clusterin / semen / dendritic / DC-SIGN / tolerance

Introduction

Clusterin is a ubiquitous glycoprotein of 75–80 kDa present in almost all tissues and body fluids. It is highly conserved among mammals showing 70–80% identity at the amino acid level. Although clusterin appears to be involved in a variety of physiological and pathological processes, such as cell death regulation, complement inhibition, Alzheimer's disease and cancer progression, its function is still controversial and enigmatic (Jones and Jomary, 2002; Wu *et al.*, 2012; Niforou *et al.*, 2014).

Clusterin was the first extracellular chaperone described (Wilson and Easterbrook-Smith, 2000). Other well-characterized extracellular chaperons are haptoglobin and α-2 macroglobulin (Yerbury *et al.*, 2005; Wyatt *et al.*, 2013a,b). Extracellular chaperones bind to stress-damaged proteins, preventing the formation and deposition of insoluble aggregates and promoting the clearance of extracellular stressed proteins by receptor-mediated endocytosis followed by lysosomal degradation (Wyatt *et al.*, 2013a,b). Failure of these mechanisms contributes to the development of the so-called protein deposition diseases such as amyloidosis, atherosclerosis, Alzheimer's or Creutzfeldt–Jakob diseases (Wyatt *et al.*, 2013a,b). Clusterin is able to interact with a variety of proteins after exposure to different stressors like heating, oxidative damage, chemical reducing agents or shear stress (Wyatt *et al.*, 2009a,b). Although the structure of clusterin has not been fully resolved, it is predicted to contain three amphipathic alpha-helices which may interact with hydrophobic regions that become surface-exposed in stressed proteins (Bailey *et al.*, 2001). The chaperone activity of clusterin is ATP-independent and results in the formation of high molecular weight complexes with misfolded proteins (Wyatt *et al.*, 2009a,b).

Clusterin is a highly glycosylated protein with 30% of its molecular mass contributed by glycans (Kapron *et al.*, 1997; Jones and Jomary, 2002). The concentration of clusterin in human blood plasma is around 100 μg/ml while the concentration in semen is at least 20-fold higher (i.e. 2–10 mg/ml), raising the question about the role of seminal plasma clusterin in reproduction (Choi *et al.*, 1990). We have previously reported that seminal plasma clusterin bears highly fucosylated Lewis X and Lewis Y type glycans which confer clusterin the ability to bind with high affinity to DC-SIGN, a C-type lectin receptor selectively expressed on dendritic cells (DCs) (Sabatte *et al.*, 2011). Intriguingly, the glycosylation pattern of serum clusterin is different from its semen counterpart, bearing mostly sialylated glycans (Kapron *et al.*, 1997). Indeed, in contrast with seminal plasma clusterin, serum clusterin does not interact with DC-SIGN (Sabatte *et al.*, 2011).

Here we show that seminal plasma clusterin not only displays a chaperone activity similar to serum clusterin, but also targets stressed proteins to DCs via DC-SIGN. Clusterin clients are then endocytosed and sorted to intracellular degradative compartments involved in antigen processing and presentation. We hypothesize that this mechanism might play a role in the induction of immune tolerance to paternal antigens required for normal pregnancy. It should be emphasized that the acquisition of female tolerance against seminal antigens requires the stimulation of an active tolerogenic response which involves the participation of both tolerogenic DCs and regulatory T cells (Tregs) (Steinman *et al.*, 2003; Aluvihare *et al.*, 2004; Moldenhauer *et al.*, 2009; Robertson *et al.*, 2013). In this regard, it has been shown that exposure of the female genital mucosa to seminal plasma triggers the expansion of Tregs in draining lymph nodes (Robertson *et al.*, 2009). Moreover, we recently reported that seminal plasma induces the differentiation of DCs into a tolerogenic profile (Remes Lenicov *et al.*, 2012). Of note, the induction of a state of tolerance against seminal antigens requires the interaction and endocytosis of seminal antigens by DCs. Our present results suggest that seminal plasma clusterin might play a role in these processes by promoting the endocytosis of stressed seminal antigens by DCs via DC-SIGN. Supporting that this novel pathway of antigen uptake might be associated with the induction of tolerance rather than immunity, we found that the interaction of seminal plasma clusterin with DC-SIGN promotes the ability of DCs to stimulate the expansion of CD25+FOXP3+ CD4+ T cells. Interestingly, a similar tolerogenic response was induced by other compounds found in semen at unusually high concentrations, such as transforming growth factor (TGF)-β and prostaglandin E2 (Robertson and Sharkey, 2001; Moldenhauer *et al.*, 2009; Robertson *et al*, 2013), further suggesting that seminal antigens picked up by DCs through DC-SIGN will induce tolerance rather than immunity.

Materials and Methods

Reagents

Catalase, Glutathione-S-transferase (GST) and Bovine Serum Albumin (BSA) were obtained from Sigma-Aldrich (USA). Antibodies against DC-SIGN were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, MD, USA (clones 120507 and 120526) and from R&D Systems (USA) (clone 120612). Polyclonal goat antibodies against clusterin were from R&D Systems (USA).

Seminal plasma samples

All protocols using human cells and semen samples were approved by the Ethical Committee of the National Academy of Medicine (Buenos Aires, Argentina). Seminal plasma samples were collected from healthy donors (aged 25–45 years) as previously described (Sabatte *et al.*, 2007). Informed consent was obtained from each patient before sperm collection. Ejaculates were obtained by masturbation under hygienic conditions, after a period of 2–4 days of sexual abstinence, and were collected in sterile containers. The samples were allowed to liquefy for 30 min at 37°C. Seminal plasma was separated from the cell fraction by centrifugation (15000 × *g*, 30 min at 4°C), and the supernatant was passed through a 0.22 μm-pore-size syringe filter (Millipore, The Netherlands) and stored at −80°C until use.

Purification of clusterin

Purification was performed by affinity chromatography as described (Sabatte *et al.*, 2011), using anti-clusterin antibodies coupled to an N-hydroxysuccinimide (NHS)-activated HiTrap column. Briefly, a pool of 5 ml of seminal plasma collected from five different donors was diluted 1/10 in phosphate-buffered saline (PBS) in the presence of a complete protease inhibitor mixture (Roche, Germany). The sample was continuously recirculated through the column, using a peristaltic pump, for 16 h at 4°C. Then, the column was washed with

100 ml of PBS and the retained material was eluted with glycine 0.1 M at pH 2.8. Purified clusterin was dialyzed twice against 1 l of PBS for 16 h at 4°C, concentrated, and finally analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (under reducing or non-reducing conditions) and Coomassie staining (LabSafe Gel blue; G-Bioscience, USA) (Supplementary Fig. S1). The concentration of clusterin stocks was determined by enzyme-linked immunosorbent assay (R&D Systems, USA).

Protein precipitation assays

Solutions of catalase (200 μg/ml), GST (400 μg/ml) and human serum (diluted 1/10 in PBS) were prepared in PBS and heated at 60°C in the presence or absence of seminal plasma or serum clusterin (100 μg/ml). The light scattering of the solution at 360 nm was measured every 1 min for 20 min in a Bio Rad SmartSpect 3000 (USA). Catalase (200 μg/ml), GST (400 μg/ml) or BSA (500 μg/ml), were incubated at 37°C for 120 min in PBS containing dithiothreitol (DTT, 20 mM), in the absence or presence of seminal plasma or serum clusterin (at concentrations of 200, 400 or 500 μg/ml, for catalase, GST and BSA, respectively). The light scattering of the solutions at 360 nm was measured every 8 min.

Cell lines

Human Raji cells, an Epstein Barr Virus-positive Burkitt's lymphoma-derived cell line, and DC-SIGN-expressing Raji cells were obtained from the AIDS Research and Reference Reagent Program, MD, USA, and grown in complete medium: RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U of penicillin/ml and 50 μg of streptomycin/ml (Gibco, USA).

Preparation of human DCs

Peripheral blood mononuclear cells were isolated from blood of healthy donors by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Sweden). Monocytes were obtained using CD14 microbeads (Miltenyi Biotec, Germany). To obtain DCs, monocytes were cultured for 5 days at a final concentration of 1×10^6 cells/ml in complete medium supplemented with 20 ng/ml of interleukin (IL)-4 and 20 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec, Germany). On Day 5, the cells were analyzed by fluorescence-activated cell sorter (FACS).

Binding of stressed proteins to DC-SIGN expressing Raji cells and DCs

Binding assays were carried out as follows: first, biotin-labeled catalase (200 μg/ml) or GST (400 μg/ml), or mixtures of clusterin (100 μg/ml) with catalase or GST were heated for 20 min at 60°C or incubated at 4°C (controls). Then, the solutions were cooled and 20 μl of each solution were added to Raji cells, DC-SIGN expressing Raji cells or DCs (5×10^5 cells in 40 μl of complete medium), and incubated for 60 min at 4°C. The cells were then washed twice with cold complete medium, stained with fluorescein isothiocyanate (FITC)-conjugated streptavidin and analyzed by flow cytometry. Binding assays were also carried out with a solution of biotin-labeled BSA (500 μg/ml) stressed by heating at 37°C for 150 min with DTT (20 mM), in the absence or presence of clusterin (500 μg/ml). In some experiments, cells were pre-treated for 30 min at 4°C with mannan (1 mg/ml), DC-SIGN blocking antibodies (10 μg/ml) or mouse immunoglobulin G as a control.

Enzymatic treatment of clusterin

Clusterin purified from seminal plasma (3–10 μg) was treated with 50 mU of α1–2 fucosidase or 0.5 mU of α1-3,4 fucosidase (Calbiochem, USA) for 3 h at 37°C in 50 mM sodium phosphate buffer (pH 5).

Flow cytometry

FITC or phycoerythrin-conjugated monoclonal antibodies (mAbs) directed to CD40, CD80, CD86, HLA-DR, CD83 and CD209 (DC-SIGN) were from BD Pharmingen (USA). In all cases isotype-matched control antibodies were used. Analysis was performed using a BD FACSCanto cytometer and BD FACSDiva software (BD Biosciences, USA).

Endocytosis assays

Mixtures of clusterin (100 μg/ml) with catalase (200 μg/ml) were heated for 20 min at 60°C. Then, 20 μl of these solutions were added to DCs (5×10^5 cells in 40 μl of complete medium) and incubated for 60 min at 4°C. Then, cells were washed twice using cold complete medium and stained with FITC-conjugated streptavidin for 15 min at 4°C. Cells were washed, incubated for 30 min at 37 or 4°C and then analyzed by flow cytometry in the presence or absence of Trypan Blue (20% v/v).

Confocal microscopy analysis

DCs were placed on poly-L-lysine–coated glass coverslips (12 mm) for 30 min at room temperature. Binding assays were carried out as follows: biotin-labeled catalase (200 μg/ml) was heated for 20 min at 60°C in the presence of clusterin (100 μg/ml), the solution was cooled, added to DCs and incubated for 60 min at 4°C. After that, cells were washed and the coverslips were covered with 100 μl of complete medium and incubated at 37°C for 15 min or 30 min to allow endocytosis. The cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min on ice. Then, cells were treated with 0.1 mM glycine in PBS and incubated with mAbs directed to early endosome antigen 1 (EEA1), lysosome-associated membrane protein 1 (LAMP-1), clusterin or DC-SIGN for 1 h, washed and stained with conjugated secondary antibodies (Jackson Immuno Research, USA). Coverslips were mounted on glass slides using Fluoromount G. Immunofluorescence images were acquired with a FluoView FV1000 confocal microscope (Olympus, USA) using a Planapo 60x 1.42 NA immersion objective.

Analysis of CD25+FOXP3+ CD4+ T cell expansion by DCs

CD4+ T cells were purified by using a CD4+ T cell isolation kit (Miltenyi). DCs (5.10^4/100 μl) were incubated with seminal plasma clusterin (40 μg/ml) or PBS and cultured for 24 h in complete medium containing 20 ng/ml of IL-4 and 20 ng/ml of GM-CSF. DCs were then washed and cultured with allogeneic CD4+ T cells (2.10^5/100 μl) for 5 additional days in complete medium into U-shaped culture plates. After this period cells were stained using antibodies directed to CD4, CD25 and FOXP3, following manufacturer's instructions (BD Biosciences, USA), and analyzed by flow cytometry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (La Jolla, CA, USA). Statistical comparisons of two groups were performed by using paired *t*-test. Statistical comparisons for multiple groups were performed by using analysis of variance followed by Bonferroni's multiple comparison post test. The *P*-values <0.05 were considered statistically significant.

Results

Seminal plasma clusterin has chaperone activity

We first analyzed whether seminal plasma clusterin was able to display chaperone activity, as was described for serum clusterin (Wilson and Easterbrook-Smith, 2000; Yerbury *et al.*, 2007; Wyatt *et al.*, 2009a,b,

2011; Wyatt and Wilson, 2010). To this aim, clusterin was purified from seminal plasma or serum samples. Its ability to prevent the precipitation of stressed-proteins was evaluated by measuring the absorbance at 360 nm.

Figure 1A–C and G shows the effect of seminal plasma clusterin (CLU) and serum clusterin (serum-CLU) on the precipitation of catalase, GST, and normal human serum, induced by heating at 60°C, while Fig. 1D–F and H shows the effect of clusterin on the precipitation of catalase, GST, and BSA, induced by treatment with DTT, as previously described (Humphreys *et al.*, 1999). The results indicated that seminal plasma clusterin significantly inhibited the precipitation of all the proteins assessed, in a similar fashion than serum clusterin. We conclude that seminal plasma clusterin has chaperone activity.

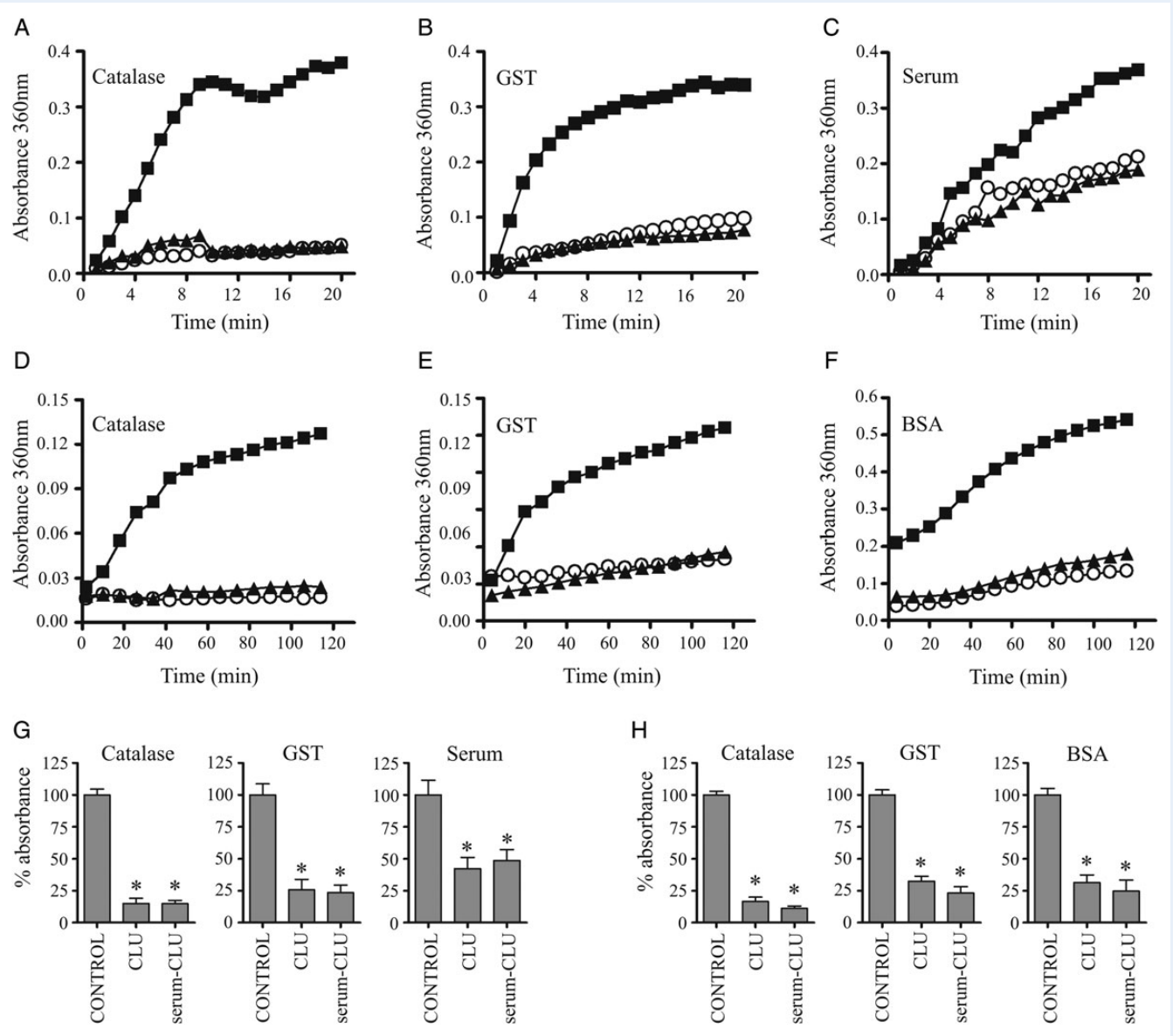

Figure 1 Seminal plasma clusterin has chaperone activity. (**A**–**C**) Catalase (200 μg/ml), GST (400 μg/ml) or human serum (diluted 1 in 10 in phosphate-buffered saline), were incubated for 20 min at 60°C in the presence or absence of seminal plasma clusterin (CLU) (100 μg/ml) or serum clusterin (serum-CLU) (100 μg/ml). (**D**–**F**) Catalase (200 μg/ml), GST (400 μg/ml) or bovine serum albumin (BSA) (500 μg/ml), were incubated for 120 min at 37°C in a buffer containing dithiothreitol (DTT) (20 mM) in the presence or absence of CLU or serum-CLU, at concentrations of 200, 400, or 500 μg/ml, for catalase, GST, and BSA, respectively. Absorbance at 360 nm was measured at different time points. Squares (■) represent controls, triangles (▲) represent proteins incubated with CLU and circles (○) represent proteins incubated with serum-CLU. Representative experiments are shown ($n = 3–6$). (**G** and **H**) Experiments in (G) and (H) were performed as described above for (A)–(C) (20 min at 60°C), and (D)–(F) (120 min at 37°C with DTT 20 mM), respectively. Results are expressed as the mean ± SEM of 3–6 experiments. The absorbance of stressed-proteins incubated with CLU or serum-CLU was expressed as the percentage of the absorbance of proteins stressed in the absence of clusterin (CONTROL). *$P < 0.01$ versus CONTROL (student's *t*-test).

Seminal plasma clusterin targets extracellular stressed proteins to DC-SIGN

We have previously reported that human seminal plasma clusterin binds to DC-SIGN on DCs with high affinity (Kd ~76 nM) (Sabatte *et al.*, 2011). We hypothesize that seminal plasma clusterin might target stressed proteins to DC-SIGN on DCs influencing the adaptive immune response to seminal antigens. To test this hypothesis we first analyzed the ability of seminal plasma clusterin to induce the binding of biotin-labeled stressed catalase to DC-SIGN-expressing Raji cells. The expression of DC-SIGN by Raji cells is illustrated in Fig. 2A. In these experiments biotin-labeled catalase (200 μg/ml) was incubated for 20 min at 60°C (stressed catalase) or 4°C with or without seminal plasma clusterin (100 μg/ml). Then, the binding of catalase to Raji cells was revealed using FITC-labeled streptavidin.

When stressed in the presence of seminal plasma clusterin, catalase efficiently binds to DC-SIGN-expressing Raji cells (Fig. 2B) but not to DC-SIGN (−) Raji cells (Fig. 2C), the binding being completely prevented by the DC-SIGN inhibitor mannan (Fig. 2D). As expected, not only stressed catalase, but also stressed GST and BSA were efficiently carried by seminal plasma clusterin to DC-SIGN (Fig. 2E–G). Serum clusterin does not interact with DC-SIGN (Sabatte *et al.*, 2011) and consequently it did not induce the binding of stressed proteins to DC-SIGN-expressing Raji cells (Fig. 2E–G).

We then analyzed the ability of seminal plasma clusterin to target stressed proteins to DC-SIGN expressed on DCs. The expression of DC-SIGN by DCs is illustrated in Fig. 3A. Seminal plasma clusterin efficiently targeted stressed catalase to DCs (Fig. 3B). Both, mannan and three different anti-DC-SIGN antibodies that recognize and block the carbohydrate recognition domain of DC-SIGN markedly inhibited the binding of stressed-catalase to DCs (Fig. 3C–F). This indicates that DC-SIGN is the main receptor for clusterin-client protein complexes on DCs. Moreover, consistent with our previous observations indicating that seminal plasma clusterin binds to DC-SIGN trough Lewis type terminal fucose motifs (Sabatte *et al*, 2011), we found that the treatment of seminal plasma clusterin with α1-3,4 fucosidase or α1-2 fucosidase inhibited its ability to target stressed-catalase to DCs (Fig. 3G and H).

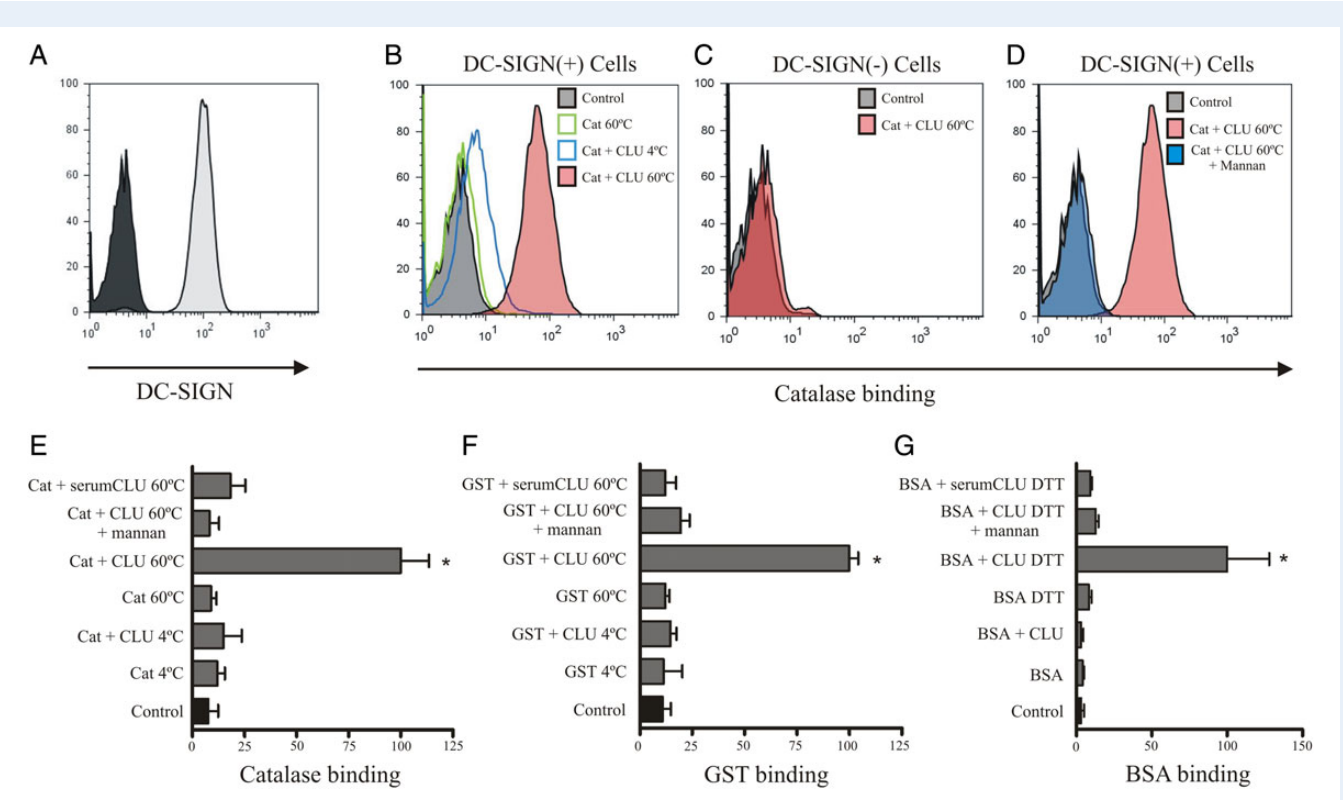

Figure 2 Clusterin targets stressed proteins to DC-SIGN. (**A**) Raji DC-SIGN (+) cells were stained using fluorescein isothiocyanate (FITC)-conjugated anti-DC-SIGN antibodies and analyzed by flow cytometry. Black histogram represent the isotype control. (**B**–**D**) Biotin-labeled catalase (200 μg/ml) was incubated for 20 min at 60 or 4°C with or without CLU (100 μg/ml). The binding of catalase to DC-SIGN (+) Raji cells (B) or DC-SIGN (−) Raji cells (C) was analyzed by incubating cells with biotin-labeled catalase/clusterin complexes for 60 min at 4°C. Cells were then washed and the binding of biotin-labeled catalase/clusterin complexes was revealed by flow cytometry using FITC-streptavidin. In (D), Raji DC-SIGN+ cells were pre-treated for 30 min at 4°C with mannan (1 mg/ml) to block DC-SIGN. Then, the binding of biotin-labeled catalase/clusterin complexes was assessed as described for (B) and (C). Representative histograms are shown ($n = 4$). (**E** and **F**) Biotin-labeled catalase (200 μg/ml) or GST (400 μg/ml) were incubated for 20 min at 60 or 4°C in the absence or presence of CLU (100 μg/ml). Then, catalase and GST were incubated for 60 min at 4°C with DC-SIGN-expressing Raji cells, pre-treated, or not, with mannan (1 mg/ml). Binding of biotin-labeled catalase or GST was revealed by flow cytometry using FITC-streptavidin. Binding of catalase stressed at 60°C in the presence of CLU was considered as 100%. Results are the mean ± SEM of three experiments (*$P < 0.01$). (**G**) Biotin-labeled BSA (500 μg/ml) was incubated for 60 min at 37°C with DTT (20 mM) in the absence or presence of CLU (500 μg/ml). Binding of biotin-labeled BSA was assessed as described in (E) and (F). Results are the mean ± SEM of three experiments (*$P < 0.01$, analysis of variance (ANOVA)).

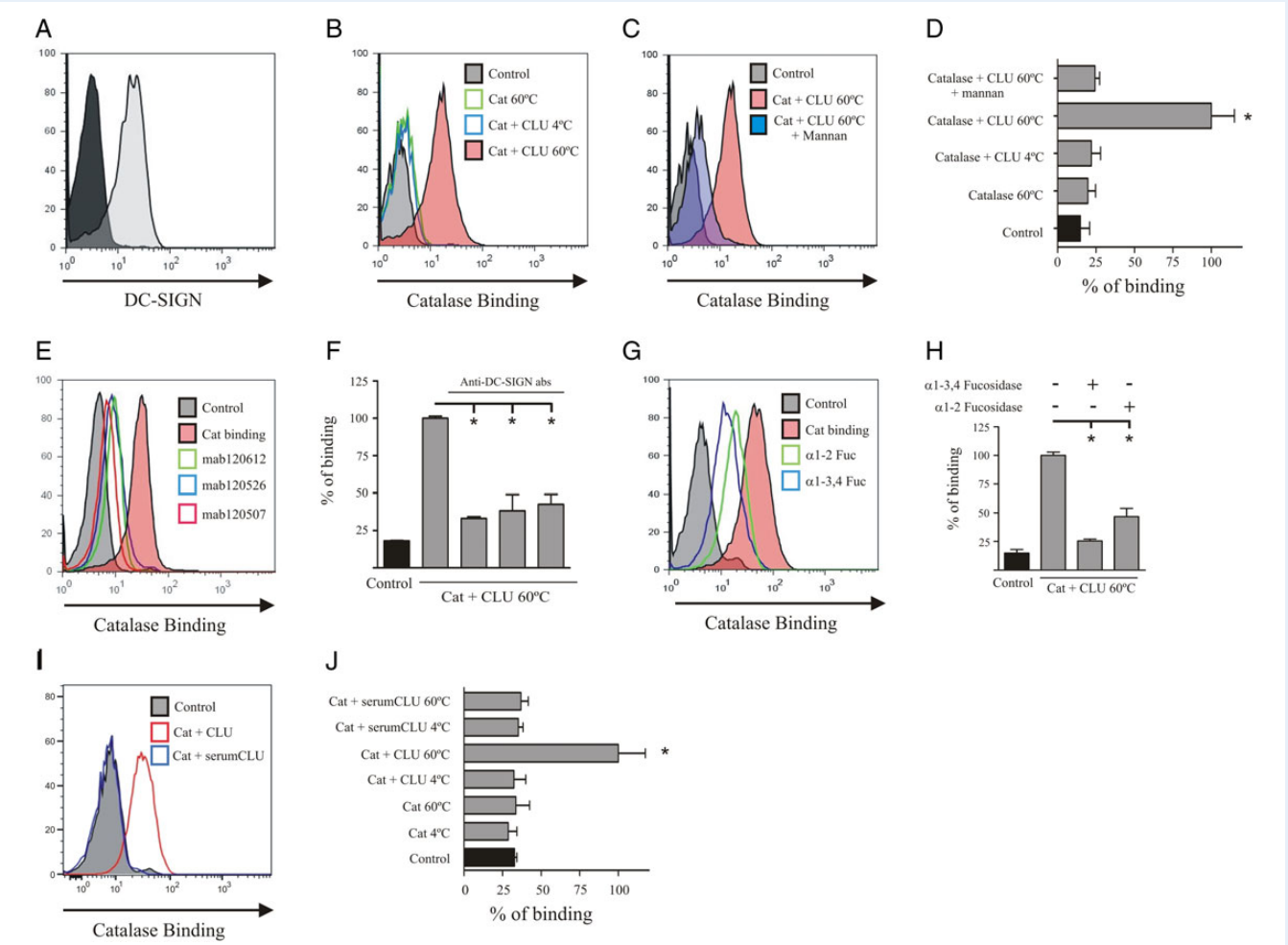

Figure 3 Clusterin targets stressed proteins to dendritic cells. (**A**) Dendritic cells (DCs) were stained using FITC-conjugated anti-DC-SIGN antibodies and analyzed by flow cytometry. Black histogram represent the isotype control (**B**–**H**). Biotin-labeled catalase (200 μg/ml) was incubated in the absence or presence of CLU (100 μg/ml) at 60 or 4°C. Then, the binding of biotin-labeled catalase to DCs was analyzed by flow cytometry using FITC-streptavidin. In (C), DCs were pre-treated, or not, with mannan for 30 min at 4°C, to block DC-SIGN. Representative histograms ($n = 3$) are shown in (B) and (C), and the mean ± SEM of three independent experiments are shown in (D). In (E) and (F), DCs were pre-treated, or not, with three different blocking antibodies directed to DC-SIGN (from left to right in F: clones 120507, 120526 and 120612). Representative histograms ($n = 3$) are shown in (E**)** and the mean ± SEM of three independent experiments are shown in (F) (*$P < 0.01$). In (G) and (H), assays were performed using biotin-labeled catalase (200 μg/ml) stressed in the presence of CLU (100 μg/ml), previously treated, or not, with α 1-3, 4 or α 1-2 fucosidase. Representative histograms ($n = 3$) are shown in (G) and the mean ± SEM of three independent experiments are shown in (H**)** (*$P < 0.01$). (**I** and **J**) Biotin-labeled catalase (200 μg/ml) was incubated in the absence (Control) or presence of CLU (100 μg/ml) or serum-CLU (100 μg/ml) for 20 min at 60°C. Then, the binding of biotin-labeled catalase to DCs was analyzed by flow cytometry using FITC-streptavidin. Representative histograms ($n = 3$) are shown in (I) and the mean ± SEM of three independent experiments are shown in (J) (*$P < 0.01$ versus control, ANOVA test).

As expected, serum clusterin was shown to be completely unable to target stressed catalase to DCs (Fig. 3I–J).

Seminal plasma clusterin induces the endocytosis of stressed proteins by DCs

Because binding to DC-SIGN results in the internalization of their ligands (Engering *et al.*, 2002; Geijtenbeek and van Kooyk, 2003; Cambi *et al.*, 2009), we next analyzed the ability of seminal plasma clusterin to induce the endocytosis of stressed proteins and the endocytic pathway followed by them. In a first set of experiments, we took advantage of the ability of the colorant trypan blue to quench the green fluorescence of extracellular proteins attached to the cell surface (Sahlin *et al.*, 1983). In these experiments, biotin-labeled catalase was stressed in the presence of seminal plasma clusterin for 20 min at 60°C and then incubated with DCs for 60 min at 4°C. Cells were then washed, stained at 4°C using FITC-conjugated streptavidin and incubated for an additional period of 30 min at 4 or 37°C, to allow catalase endocytosis. Trypan blue almost completely quenched fluorescence in cells cultured at 4°C (Fig. 4A) but only slightly quenched fluorescence in cells cultured at 37°C (Fig. 4B), indicating that catalase was efficiently internalized. As expected, studies performed by confocal microscopy revealed the colocalization of clusterin and catalase inside DCs (Fig. 4C).

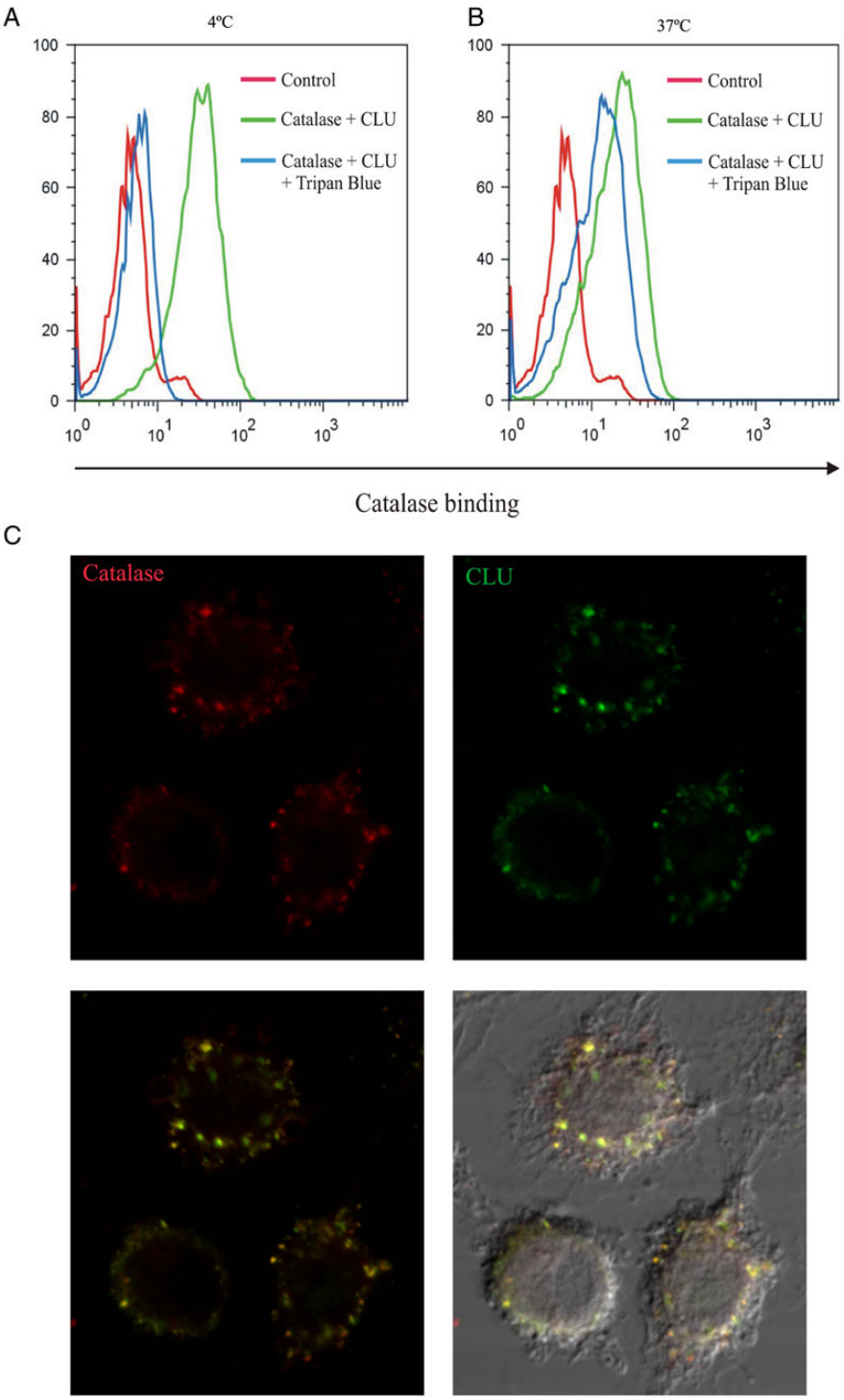

Figure 4 Clusterin-client complexes are endocytosed by DCs. Biotin-labeled catalase (200 μg/ml) was incubated at 60°C for 20 min with CLU (100 μg/ml). Then, the solution was cooled and incubated with DCs for 60 min at 4°C. Cells were washed and stained using FITC-conjugated streptavidin. After washing, cells were cultured for an additional period of 30 min at 4°C (**A**) (to prevent endocytosis) or at 37°C (**B**) (to allow endocytosis). Finally, cells were analyzed by flow cytometry in the presence or absence of trypan blue. Representative histograms ($n = 3$) are shown. In (**C**), endocytosis of stressed catalase induced by CLU was analyzed by confocal microscopy. Biotin-labeled catalase (200 μg/ml) was incubated at 60°C during 20 min with CLU (100 μg/ml). Clusterin-catalase complexes were then incubated with DCs for 60 min at 4°C. After washing, cells were treated with fluorescent-labeled streptavidin for 30 min at 4°C. Finally, cells were washed, incubated for 15 min at 37°C to allow endocytosis, fixed and stained using anti-clusterin antibodies and fluorescent-labeled secondary antibodies. Colocalization of catalase and CLU was analyzed by confocal microscopy. Representative images are shown at ×300 magnification ($n = 5$).

To define the fate of endocytosed proteins targeted by clusterin, the endocytic route of biotin-labeled catalase was followed using endosome specific markers that distinguish between early and late endosomes/lysosomes: early EEA1 and LAMP-1, respectively. As shown in Fig. 5A, catalase colocalized with DC-SIGN at the cell surface at 5 min, but not at 30 min of endocytosis (Fig. 5A), the time at which most of the catalase exhibited an intracellular localization. Co-localization of catalase with EEA1 and LAMP-1 was evident at 15 and 30 min of endocytosis, respectively (Fig. 5B and C), suggesting that catalase is sorted to late endosomes for degradation.

Seminal plasma clusterin improves the ability of DCs to promote the expansion of CD25+FOXP3+CD4+ T cells

Considering previous observations indicating that DC-SIGN-mediated signaling promotes the differentiation of DCs into a tolerogenic profile (Gringhuis *et al.*, 2007, 2009), we analyzed whether besides the ability of seminal plasma clusterin to interact with stressed proteins and target them to DC-SIGN, it might also be capable of modulating the phenotype and function of DCs. To address this aim, DCs were cultured for 24 h with or without lipopolysaccharide (LPS), in the absence or presence of seminal plasma clusterin, and the phenotype of DCs was then analyzed by flow cytometry. Figure 6A shows that seminal plasma clusterin did not modify the expression of HLA-DR, CD83, CD80, CD86, CD40 and DC-SIGN in resting or LPS-treated DCs. Additional experiments were performed to analyze whether seminal plasma clusterin was able to modulate the expansion of regulatory T cells (CD25+FOXP3+CD4+ T cells) induced by DCs in the mixed leukocyte reaction (MLR). To this aim, DCs were incubated with or without seminal plasma clusterin (40 μg/ml) for 24 h, washed and cultured with allogeneic CD4+ T cells for 5 days, and the presence of regulatory T cells was analyzed by flow cytometry.

As shown in Fig. 6B and C, seminal plasma clusterin stimulated the expansion of CD25+FOXP3+CD4+ T cells in the MLR. No effect was observed when CD4+ T cells were cultured with seminal plasma clusterin (40 μg/ml) without allogeneic DCs, either in the absence (not shown) or presence of anti-CD3 (1.2 μg/ml) and anti-CD28 (1 μg/ml) antibodies, used to induce T cell activation and proliferation: percentage of CD25+FOXP3+CD4+ T cells = 14.4 ± 4.2 versus 16.9 ± 3.9, for T cells cultured with or without seminal plasma clusterin, respectively ($n = 3$). This suggests that seminal plasma clusterin stimulates the expansion of CD25+FOXP3+CD4+ T cells by promoting the acquisition of a tolerogenic profile by DCs.

Discussion

In this study we showed that human seminal plasma clusterin has chaperone activity and targets stressed proteins to DC-SIGN. Binding of clusterin-client complexes to DC-SIGN was observed using concentrations of clusterin 50 to 100 fold lower than those normally found in semen (Choi *et al.*, 1990). DC-SIGN was shown to be the main receptor for clusterin-client complexes on DCs, in fact, mannan and three different blocking antibodies directed to DC-SIGN almost completely prevented the binding of clusterin-client complexes to DCs. After binding, these complexes were internalized by DCs into EEA1-positive compartments and then sorted into LAMP1-positive late endosomes, a pathway employed by DCs to process and present internalized antigens to prime T cells.

Clusterin was the first extracellular chaperone described (Humphreys *et al.*, 1999). Extracellular chaperones bind to stressed misfolded proteins inhibiting the formation of insoluble aggregates, maintaining their solubility and preventing their inherent toxic effects (Wyatt *et al.*, 2013a,b). Moreover, extracellular chaperones promote the clearance of misfolded proteins delivering them to specific cellular receptors for endocytosis and intracellular degradation (Wyatt *et al.*, 2013a,b). In this regard, it has been shown that clusterin-amyloid β-peptide complexes bind to the receptor megalin (also called LRP2) being subsequently internalized and degraded (Hammad *et al.*, 1997). However, in contrast with the ubiquitous expression of clusterin, megalin is only expressed at the proximal tubules of the kidney, in the choroid plexus epithelium and in ependymal cells lining the brain ventricles (Lundgren *et al.*, 1997). On the other hand, scavenger receptors appear also to be able to mediate the clearance of systemic clusterin-client complexes (Wyatt *et al.*, 2011). The function of seminal plasma clusterin as an extracellular chaperone appears to be quite different from its serum counterpart. First, the chaperone activity of seminal plasma clusterin is confined to two major scenarios, the male and the female genital tracts. Second, because DC-SIGN is selectively expressed by DCs, which express the unique ability to activate naïve T cells (Guermonprez *et al.*, 2002), the most important function of seminal plasma clusterin might not be related to the clearance of stressed proteins from the extracellular space, but rather it might be related to the targeting of stressed proteins to DC-SIGN in order to promote antigen presentation. In this regard, it should be emphasized that antigen presentation results not only in immunity, but also in tolerance, depending on the functional profile of DCs (Steinman *et al.*, 2003).

Previous studies have shown that DC-SIGN not only mediates the internalization of extracellular ligands, but also modulates the functional profile of DCs (Geijtenbeek and Gringhuis, 2009). A large body of evidence indicates that the interaction of DC-SIGN with a variety of ligands, such as measles virus (Mesman *et al.*, 2014), *Helicobacter pylori* (Gringhuis *et al.*, 2009), myelin oligodendrocyte glycoprotein (Garcia-Vallejo *et al.*, 2014), and therapeutic preparations of immunoglobulins such as intravenous gammaglobulin (IVIG) (Anthony *et al.*, 2011), promotes the acquisition of a tolerogenic profile by DCs. Recent studies, on the other hand, have revealed that ligation of DC-SIGN could also promote the differentiation of CD4+ T cells into a *T follicular helper* (Gringhuis *et al.*, 2014a,b) or *T helper 2* profile (Gringhuis *et al.*, 2014a,b). In line with these observations we found that, even when seminal plasma clusterin did not modify the phenotype of DCs evaluated by the expression of HLA-DR, CD80, CD86, CD40, DC-SIGN and CD83, it stimulates the ability of DCs to expand CD25+FOXP3+CD4+ T cells, a phenotype usually associated with regulatory T cells (Sakaguchi *et al.*, 2010). This suggests that antigens carried out by seminal plasma clusterin to DC-SIGN might induce tolerance rather than immunity.

Besides clusterin, human seminal plasma contains other DC-SIGN ligands: galectin-3 binding protein, prostatic acid phosphatase, protein C inhibitor (Clark *et al.*, 2012), and mucin 6 (Stax *et al.*, 2009). None of these ligands display a chaperone-like activity; however, all of them might promote the differentiation of DCs into a tolerogenic profile by interacting with DC-SIGN. The acquisition of a tolerogenic profile by DCs induced by DC-SIGN ligands might be important in two distinct scenarios. In the female genital tract, it might induce maternal tolerance to male alloantigens. Moreover, it could also play a role in the male genital

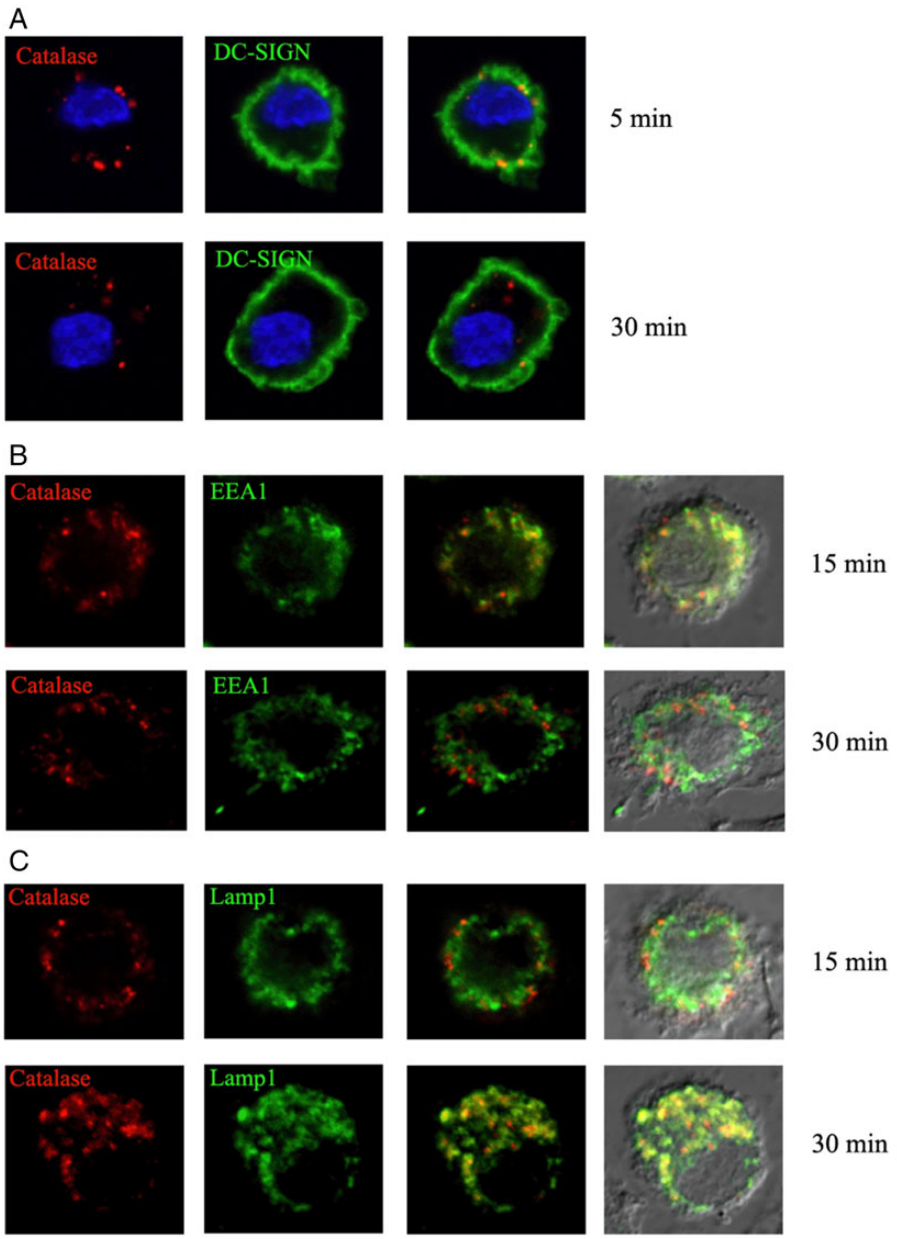

Figure 5 Analysis of clusterin-client containing compartments in DCs. Biotin-labeled catalase (200 μg/ml) was incubated at 60°C for 20 min with CLU (100 μg/ml). Clusterin-catalase complexes were then incubated with DCs for 60 min at 4°C, washed and stained using fluorescent-labeled streptavidin. After this period, cells were incubated at 37°C for 5 min (**A**, upper panel), 15 min (**B** and **C**, upper panels) or 30 min (A, B, and C, lower panels) to allow endocytosis, fixed and stained using antibodies directed to DC-SIGN (A), endosome antigen 1 (EEA1) (B) or lysosome-associated membrane protein 1 (LAMP1) (C). Nuclear counterstain with DAPI was used in (A). Representative images are shown at ×300 magnification ($n = 3–5$).

tract. It is well known that neoantigens in the semen are produced during puberty, long after the establishment of systemic self-tolerance (Kourilsky and Claverie, 1989). However, immune response against these neoantigens is not evoked (Fijak and Meinhardt, 2006). Considering that in the mammalian reproductive tract clusterin is produced not only by the epididymis, seminal vesicle, and prostate, but also by the testis (Sylvester *et al.*, 1991; O'Bryan *et al.*, 1994; Ahuja *et al.*, 1996), we speculate that it might promote the acquisition of a tolerogenic profile by DCs contributing to testicular immune privilege.

The tolerogenic properties of semen are not only related to the presence of clusterin and other DC-SIGN ligands. Semen contains huge concentrations of regulatory T cell-inducing agents such as TGF-β and prostaglandins (Robertson *et al.*, 2002; Remes Lenicov *et al.*, 2014). Moreover, Robertson and co-workers have shown that exposure of the female genital mucosa to semen promotes the expansion of CD25+ FOXP3+CD4+ Tregs in the lymph nodes draining the uterus, favoring tolerance to male alloantigens in mice (Guerin *et al.*, 2011; Robertson *et al.*, 2013). Interestingly, this response is induced even in the absence

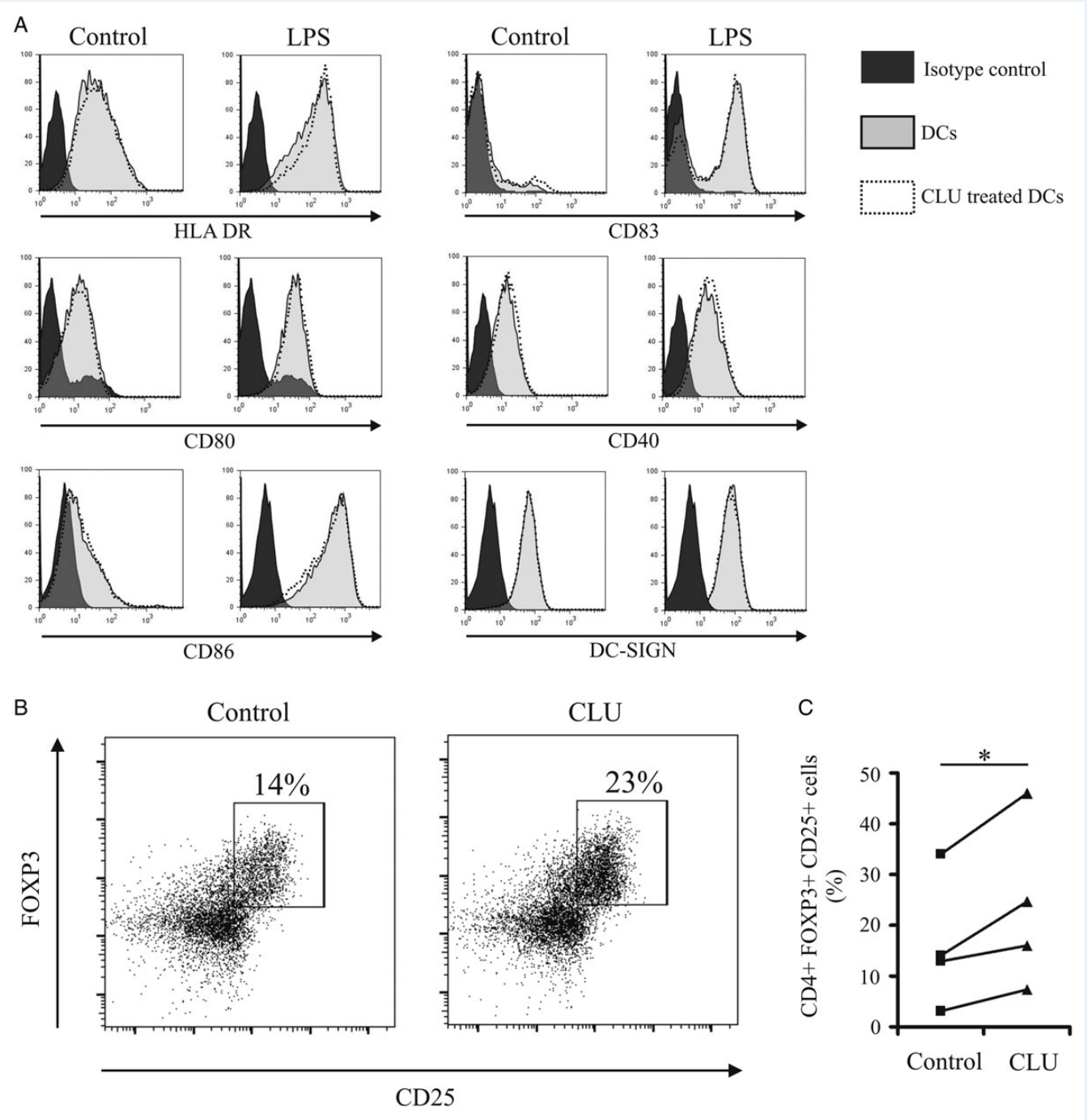

Figure 6 Clusterin stimulates the ability of DCs to expand CD25+FOXP3+CD4+ T cells. (**A**) DCs (1×10^6/ml) were cultured for 24 h in the absence (gray histograms) or presence of CLU (40 μg/ml) (dotted histograms), with or without lipopolysaccharide (LPS) (10 ng/ml). The expression of HLA-DR, CD80, CD86, CD83, CD40 and DC-SIGN was then analyzed by flow cytometry. Isotype controls are shown in black. Representative histograms ($n = 3$) are shown. (**B** and **C**) DCs (5×10^4/100 μl) were incubated with or without CLU (40 μg/ml) for 24 h, washed and cultured with allogenic T cells (DC: T cell ratio = 1:4), as described in Materials and Methods. After 5 days the percentage of CD25+FOXP3+CD4+ T cells was determined by flow cytometry. In (B), a representative dot plot is shown. In (C), the results from four independent experiments using different cell donors are shown (* = $P < 0.05$, student's *t*-test).

of conception, thus suggesting an important role for semen as a Treg inducer (Moldenhauer *et al.*, 2009).

Seminal plasma is more than just a spermatozoa carrier. It induces strong immunomodulatory effects at the female genital tract. Deposition of semen in female reproductive mucosa not only promotes the expansion and recruitment of regulatory T cells, but also triggers the activation of an acute and strong inflammatory response leading to the production of inflammatory cytokines and chemokines and the recruitment of large

numbers of neutrophils and DCs (Robertson *et al.*, 2006; Sharkey *et al.*, 2007, 2012). This inflammatory response might result in the generation of oxygen free radicals and the oxidative damage of semen proteins at the female genital tract. In this scenario, the ability of seminal plasma clusterin to target seminal proteins to DC-SIGN as well to promote a tolerogenic response against male antigens would be strengthened.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

A.M. designed and performed experiments, E.D. and F.R.L. performed experiments, A.C. was involved in the conception and coordination of the project, C.J. was involved on the acquisition and analysis of confocal microscopy data, A.V. and J.R. were involved on the acquisition and analysis of flow cytometry data, S.S. performed experiments, J.G and J.S. were involved in the conception and coordination of the project, designed the experiments and wrote the manuscript. All authors revised and approved the final manuscript.

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Conflict of interest

The authors have no financial conflicts of interest.

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