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ORIGINAL RESEARCH

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Clusterin protects mature dendritic cells from reactive oxygen species mediated cell death

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ABSTRACT

Dendritic cells (DCs) play a key role in the induction of the adaptive immune response. They capture antigens in peripheral tissues and prime naïve T lymphocytes, triggering the adaptive immune response. In the course of inflammatory processes DCs face stressful conditions including hypoxia, low pH and high concentrations of reactive oxygen species (ROS), among others. How DCs survive under these adverse conditions remain poorly understood. Clusterin is a protein highly expressed by tumors and usually associated with bad prognosis. It promotes cancer cell survival by different mechanisms such as apoptosis inhibition and promotion of autophagy. Here, we show that, upon maturation, human monocyte-derived DCs (MoDCs) up-regulate clusterin expression. Clusterin protects MoDCs from ROS-mediated toxicity, enhancing DC survival and promoting their ability to induce T cell activation. In line with these results, we found that clusterin is expressed by a population of mature LAMP3+ DCs, called mregDCs, but not by immature DCs in human cancer. The expression of clusterin by intratumoral DCs was shown to be associated with a transcriptomic profile indicative of cellular response to stress. These results uncover an important role for clusterin in DC physiology.

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Cancer; clusterin; dendritic cells; mregDC; Ros

Introduction

Dendritic cells (DCs) play a central role in the activation and regulation of the adaptive immune response.[1–3] In the context of inflammatory processes DCs are activated by different stimuli inducing their phenotypic maturation and migration to the draining lymph nodes, where they present antigens to naïve T cells.[4,5] Upon maturation, DCs survive for 4–5 days, an appropriate period of time required for an efficient interaction with T lymphocytes. After this period, they die by apoptosis.[6,7] The survival time of mature DCs influences the course of the adaptive immune response. An increased survival of mature DCs can trigger autoimmunity, while an accelerated cell death compromises the efficiency of the adaptive immune response.[7,8] In both, peripheral tissues and secondary lymphoid organs DCs face different cellular stress conditions capable of compromising their viability, such as hypoxia, low pH and/or high concentrations of reactive oxygen species (ROS). However, the molecular mechanisms that regulate DC survival have been poorly studied.[6,7,9,10]

Clusterin is a glycoprotein secreted by different cell types, found in almost all tissues and body fluids.[11,12] At the cellular level, clusterin is expressed in the nucleus, in the cytoplasm, or can be secreted to the extracellular medium.[13] Different clusterin glycoforms with contrasting properties have been characterized.[14,15] An important role for clusterin has been clearly demonstrated in processes as dissimilar as the inhibition of complement activation, the regulation of apoptosis and the removal of misfolded proteins from the extracellular space.[16,17] Clusterin expression is deregulated in different pathological processes, such as Alzheimer's disease, myocardial infarction, ocular diseases and cancer.[18,19] The expression of clusterin in neoplastic cells is usually associated with worse cancer prognosis and resistance to chemotherapy and radiotherapy.[20] Different mechanisms might explain the protumoral activity of clusterin among them, the best characterized is the inhibition of cancer cell apoptosis.[21–24]

It is well known that DC maturation is associated to an increased production of ROS. In the present study we show that maturation of MoDCs is also associated to an increased expression of clusterin which prevent ROS mediated DC death, enabling an efficient activation of T cells. Consistent with these observations performed in vitro, we found that clusterin expression is restricted to intratumoral DCs expressing a mature phenotype. Our observations reveal an unexpected role for clusterin in the function of DCs and the control of the adaptive immune response.

Materials and methods

Dendritic cell stimulation and clusterin detection

Blood from healthy donors was obtained from the blood bank of the "Hospital de Clínicas José de San Martín", Buenos Aires University (Argentina) following the institution ethical

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guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare). Monocytes were obtained using CD14 microbeads (Miltenyi Biotec). To obtain MoDCs, monocytes were cultured for 5 days at a final concentration of 1×10^6 cells/ml in RPMI-1640 medium supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin containing 10% of inactivated-fetal bovine serum (Sigma-Aldrich) (complete medium), 20 ng/ml of interleukin 4 (IL-4) and 20 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec). After 5 days, the cells were analyzed by flow cytometry. BDCA1+ human DC were isolated from PBMCs using the CD1c+ (BDCA1+) DC Isolation kit (Miltenyi Biotec), counted and cultured in complete medium prior to use.

Cells were stimulated with ultrapure E. coli LPS (10 ng/ml, Sigma-Aldrich), Pam3csk4 (1 ng/ml, Sigma-Aldrich), CpG (3 µg/ml, Sigma-Aldrich), ManLam (5 ug/ml, Sigma-Aldrich), TNF-α (10 ng/ml, Miltenyi Biotec) or HMGB1 (10 µg/ml, BioLegend) for different periods of time. MoDCs were treated with BAY-117082 (3 µg/mL, Sigma-Aldrich) or N-Acetyl Cysteine (NAC, 10 mM, Cayman Chemical) one hour before stimulation with LPS (10 ng/ml). MoDCs were also cultured alone or with allogeneic T cells (ratio of 1:2, 1:4 or 1:8) for 48hs in complete medium, using U-shaped culture plates. For exposure to acidic pH, MoDCs were incubated in RPMI medium adjusted to pH 6.5 by the addition of isotonic HCl, for 90 minutes. Then, cells were washed with complete RPMI medium at pH 7.4 and cultured in RPMI medium at pH 7.4 for 48 hours. Necrotic cells were obtained by exposing HeLa cells to five freeze-thaw cycles. In some experiments MoDCs were pre-incubated with recombinant clusterin 10–100 ng/ml (Biovendor R&D).

Clusterin from supernatants and cell lysates was measured by ELISA according to manufacturer's instructions (Human Clusterin DuoSet ELISA, R&D Systems). Clusterin mRNA was measured by real-time PCR. Total RNA was obtained from 3×10^5 cells using RNEasy kit (QIAGEN) and treated with DNAse for 15 min (SIGMA). Reverse transcription was carried out using M-MLV reverse transcriptase (SIGMA) according to the manufacturer's instructions. Briefly, 500 ng of RNA were incubated for 50 min at 37°C in the presence of 150 ng of random hexamer primers (Thermo Fisher), and 10 mM dNTP mix. cDNA was amplified using the Applied Biosystems SYBR™ Select Master Mix (Thermo Fisher) with the following primers: CLU_Fw: GAGCTCCAGGAAATGTCCAATCAG, CLU_Rev: CCTCT CATTTAGGGCATCCTCTTC. Cycle thresholds (Ct's) were normalized to the Ct of GAPDH, and fold enrichments were calculated as compared with the values from control cells.

Clusterin knock down

Clusterin knock down was achieved using shRNA carrying lentiviruses as previously described.[25] In brief, 6×10^5 HEK293T cells were seeded on a flat-bottom 6-well plate. 24hs later, cells were transfected with a mix of 1 µg pCMV-dR8.2 DVpr (RRID:Addgene_8455), 1,6 µg of the target's specific shRNA (MISSION shRNA, Sigma-Aldrich) in the pLKO.1 backbone, and 0,4 µg pCMV – VSV-G per well, using X-tremeGENE HP DNA transfection reagent (Roche), following the manufacturer's recommendations. 24 hrs later, medium was replaced, and supernatants containing lentiviral particles were collected at 48 and 72 hrs after transfection, precleared by centrifugation, aliquoted, and stored at – 80°C. Virus-like particles containing the simian immunodeficiency Vpx protein were generated in an analogous manner using a mix of 2,6 µg pSIV3+ plasmid and 0,4 µg pCMV – VSV-G. Three different clusterin shRNA carrying lentivirus were generated and called lentivirus 1 (LV1: TTGCTCCTGC ATGCAACTAAT, shRNA: TRCN0000304143), lentivirus 2 (LV2: GCTAAAGTCCTACCAGTGGAA, shRNA: TRCN0000300767) and lentivirus 3 (LV3: GCTAAAGTCC TACCAGTGGAA, shRNA: TRCN0000078611).

Monocytes were transduced with the corresponding lentiviral vector together with virus-like particles containing Vpx, in the presence of 20 ng/ml of interleukin-4, 20 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec). After 3 days, transduced cells were selected by the addition of 3 µg/ml puromycin. On day 5, live cells were separated from dead cells by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare).

Clusterin over-expression

To generate a lentiviral expression vector for human Clusterin, the open reading frame was amplified from a HeLa cDNA library by PCR using the indicated primers (Clu_Fw: GAGTCGCCCGGGGGGGGATCCGCCACCATGATGAAG-ACTCTGCTGCTGT; Clu_Rv: GCAGGTCGACTCTAG AGTCGCGGCCGCTCACTCCTCCCGGTGCTTTTT). The PCR product was then gel purified (NEB, T1020) and assembled with pHRSIN-pSFFV-GFP-PGK-Puro[26] digested with BamHI and NotI using HiFi Assembly (NEB, E2621). All constructs generated for this study were verified by Sanger sequencing (Source BioScience).

Dendritic cell maturation and mixed leukocyte reaction

10^5 scramble and CLU-KD MoDCs were cultured in 100 µl of complete medium into 96 well plates and treated with LPS (10 ng/ml). After 18 hrs, the cells were collected and analyzed by flow cytometry. The cell supernatants were harvested and the concentration of IL-12, IL-6, IL-10 and TNF-alpha were measured by sandwich ELISA according to manufacturer's instructions (BD Biosystems). CD4+ T cells were isolated using magnetic beads (Miltenyi Biotec), following the manufacturer's instructions (% purity > 94%). Isolated CD4+ T cells (1×10^7 cells/ml) were labeled with 5 µM CFSE (Molecular Probes, Invitrogen) in PBS for 5 min at 37°C. Cells were washed and plated (2×10^5/200 ml) in 96 well plates. Allogeneic scramble and CLU-KD MoDCs were counted and added to lymphocytes using a DC/CD4+T cell ratio of 1:4. After 5 days of culture, cells were harvested and CFSE dilution was assessed by flow cytometry. Quantification of CD4+ T cell proliferation was analyzed by determining the fraction of T cells that diluted CFSE dye.

Measurement of cell death

The assay was carried out using an apoptosis detection kit (BD Biosciences) following the manufacturer´s instructions. Briefly, cells were harvested, washed and incubated with FITC conjugated AnnexinV in staining buffer during 60 minutes. Then, the cells were stained with propidium iodide (1 µg/ml) for 5 minutes and analyzed by flow cytometry.

For the quantification of total viable cells, 20 µl of cell suspension was mixed with 20 µl of trypan blue 0.4% solution (Sigma-Aldrich). The cells were counted using a Neubauer chamber and the percentage of live and death cells was calculated.

Detection of reactive oxygen species (ROS)

The oxidative condition of the cells was assessed using the mitochondrial probe MitoSOX ™ (Thermo Fisher), according to the manufacturer's instructions. Briefly, the cells were stimulated or not with LPS for 1–4 hours. Subsequently, the cells were incubated for 10 minutes at 37°C in the dark with 5 µM of the mitochondrial probe and analyzed by flow cytometry.

Statistics

Statistical comparisons were performed by using paired Student's t-test or ANOVA. Non parametric data was evaluated using Mann–Whitney U test. The p values < 0.001(***), <0.01 (**) and < 0.05 (*) were considered statistically significant.

Results

In a first set of experiments, we evaluated clusterin expression in human immature and mature DCs. We stimulated human BDCA1+ blood DCs by LPS and clusterin concentration was measured in cell supernatants by ELISA. As shown in Figure 1a, LPS stimulation induced clusterin secretion. A similar observation was done using human monocyte-derived DCs (MoDCs) (Figure 1b). As clusterin can be also expressed intracellularly, we measured the concentration of clusterin in lysed MoDCs, and found a clear up-regulation in LPS-stimulated cells (Figure 1c). As expected, clusterin mRNA was upregulated in mature MoDCs (Figure 1d). Considering the critical role that *NFκB* plays in the maturation of DCs, we analyzed the effect of the *NFκB* inhibitor BAY 11–7082 on clusterin production. A marked reduction in clusterin secretion was observed when the cells were pre-treated with BAY 11–7082 (Figure 1e). We then analyzed the kinetics of clusterin expression after LPS stimulation of MoDCs. As shown in Figure 1f, both intracellular (IC) and secreted clusterin reached a maximum 48 hrs after stimulation.

Clusterin is synthesized in the endoplasmic reticulum where it is folded into a ~ 60kDa single-chain precursor protein. In the late Golgi compartment, the precursor is cleaved to generate the active form of clusterin composed by two (α and β) chains, forming a heterodimeric glycoprotein that migrates as a ~ 40kDa band in western blots.[27] As shown in Figure 1g, the stimulation by LPS induced not only the upregulation of total clusterin, but also the production of the mature form of the protein. This ~ 40 kDa form of clusterin is found in the cellular cytosol and is also secreted to the extracellular compartment. Other clusterin isoforms characterized by different molecular weights[13,28] were not detected. In addition to LPS, different microbial stimuli that promote DC maturation were shown to induce clusterin secretion (Figure 1h). It was also induced by co-culturing MoDCs with allogenic CD4 and CD8 T cells (Figure 1i) and by different non-microbial stimuli and conditions known to activate DCs such as low pH,[29] pro-inflammatory cytokines, necrotic cells or HMGB1[30] (Figure 1j).

To gain insight into the expression of clusterin by human DCs in vivo, we analyzed three published single cell transcriptomics data sets obtained from different human tumors. First, we analyzed clusterin expression by intratumoral DCs in a data set published by Cheng S. et al. We analyzed single cell RNAseq data of DCs obtained from patients with 8 different tumor types.[31] As shown in Figure 2a,b, clusterin expression is clearly restricted to the population known as mregDCs (also called LAMP3+DCs).[32,33] Remarkably, this population shows the highest expression of maturation markers, such as, LAMP3, CCR7, CD83, BIRC3 and MARCKSL1 (Figure 2c,d) suggesting that, in line with our *in vitro* results, clusterin expression is associated to the maturation of intratumoral DCs. Similar results were found when we analyzed published data sets from DCs infiltrating non-small cell lung cancer (Figure 2e, h)[33,34] and intratumoral DCs from hepatocarcinoma samples (Figure 2i,l).[35] Using the same strategy, we also analyzed clusterin expression by mregDCs in non-tumoral tissues. Interestingly, clusterin was also expressed by tonsil mregDCs (Suppl Figure S1) suggesting that clusterin expression might be associated with the maturation of DCs in different settings.[36]

Not only DCs but also monocyte-derived macrophages and monocytes enhanced clusterin production upon treatment with LPS. Monocyte-derived macrophages obtained by treatment with either GM-CSF or M-CSF released clusterin upon activation by LPS (Suppl Figure S2A and B). Similar results were observed when intracellular clusterin was evaluated (unpublished results). Activation of freshly purified monocytes also resulted in the stimulation of clusterin secretion (Suppl Figure S2C).

To evaluate the role of clusterin on DC function, we knocked down clusterin in MoDCs using lentiviruses carrying a shRNA for clusterin. We used a scramble lentivirus carrying a non-relevant shRNA as a control. We constructed three lentiviruses (LV1, LV2 and LV3) using three different shRNA for clusterin. As shown in Figure 3a, all three lentiviruses efficiently down regulate clusterin expression in resting or activated MoDCs, being LV1 and LV3 the most efficient vectors. We used LV1 and LV3 for the experiments showed below.

As shown in Figure 3b, clusterin knock down DCs (CLU-KD DCs) were shown to be able to up-regulate maturation markers and to produce cytokines such as IL-12, IL-10, TNF-α and IL-6 after overnight incubation with LPS in a comparable fashion with control DCs. However, when scramble and CLU-KD DCs were cocultured with allogenic T cells during 5 days and T cell proliferation was evaluated, a profound inhibition in the proliferation of allogeneic T cells was observed in LPS treated CLU-KD DCs compared with untreated CLU-KD DCs or with scramble LPS treated DCs (Figure 4a,b). As expected, the release of IFN-γ and IL-2 was markedly impaired in mixed cultures when LPS treated CLU-KD DCs were used as antigen-presenting cells (Figure 4c,d).

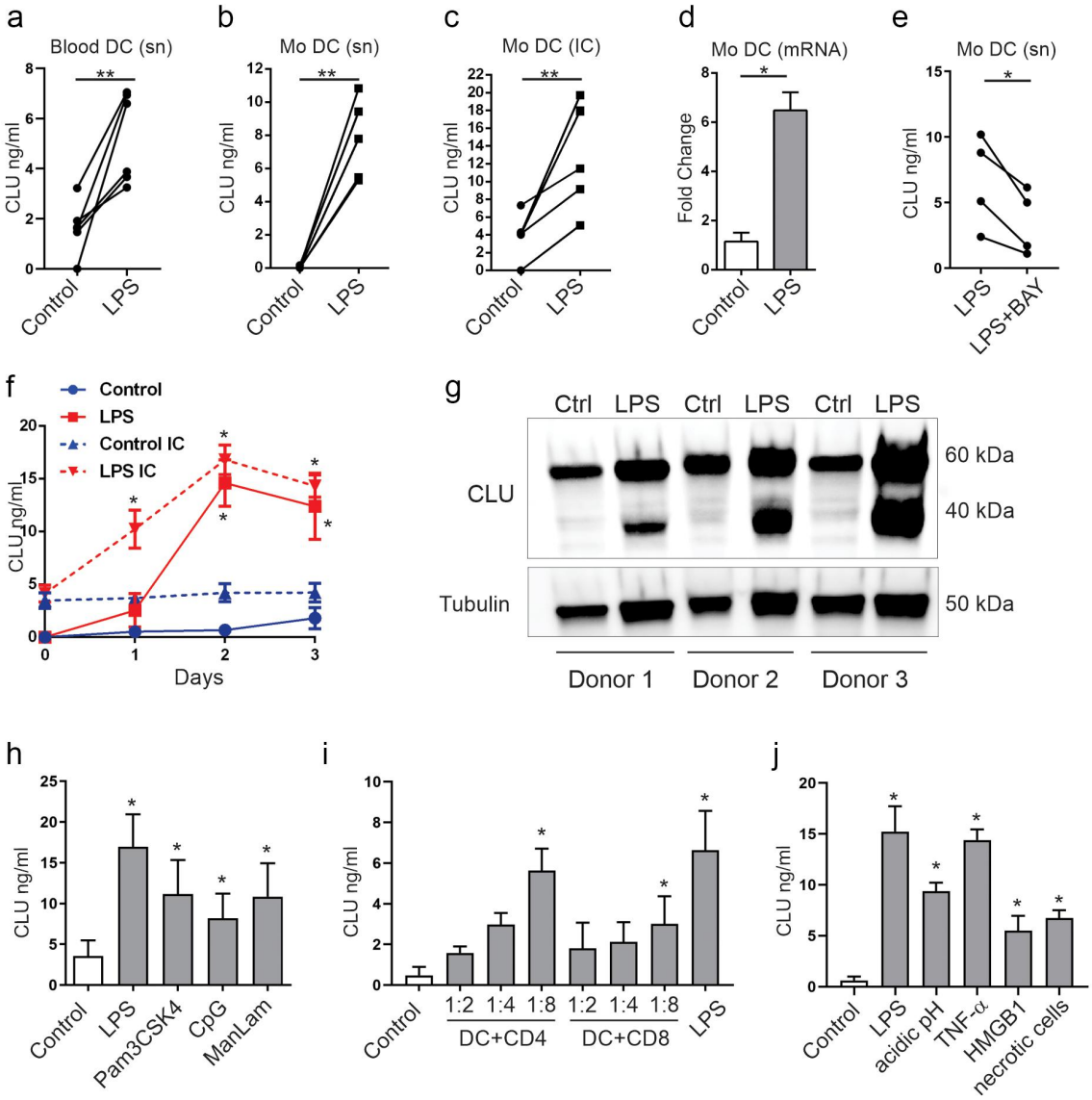

Figure 1. Dendritic cell maturation triggers clusterin expression. DCs were cultured in the absence (control) or presence of LPS (10 ng/ml). On a and b, clusterin concentration was measured by ELISA in the supernatant (sn) of BDCA1+ human DCs isolated from blood (Blood DC) and monocyte-derived DCs (MoDC), respectively. On c and d, MoDCs were lysed and clusterin concentration was measured in cell lysates (intracellular or IC) by ELISA and real-time PCR, respectively. e: clusterin secretion by LPS stimulated MoDCs, treated or not with BAY 11-7082 (3 µg/ml) was evaluated by ELISA. f: clusterin concentration in the supernatant (circles and squares) and in the cell lysates (triangles, IC = intracellular) of MoDCs stimulated or not with LPS at different time points. On g, MoDCs were treated or not (Ctrl) with LPS and the expression of clusterin was analyzed by western blot. Monocytes were isolated from 3 healthy donors. The precursor pre-protein is seen at ~ 60 kDa and the mature protein is seen at ~ 40 kDa. Tubulin was used as loading control (lower panel). On h, the concentration of clusterin in the supernatant of MoDCs stimulated with different compounds is shown. On i, MoDCs were co-cultured with CD4+ or CD8+ T cells at different cell ratios, and clusterin concentration was measured on cell supernatants. MoDCs cultured alone were used as negative control (control) and LPS as positive control (LPS). On J, MoDCs were exposed at pH 6.5, treated with TNF-α (10 ng/ml), HMGB1 (10 µg/ml) or necrotic HeLa cells (1/1 ratio), and clusterin concentration was measured in cell supernatants after 48 hs. MoDCs cultured alone were used as negative control (control) and LPS as positive control (LPS). On d, f, h, i and j, the data represent the arithmetic means ± the SD of 3-6 independent experiments carried out in triplicate, $*p < 0.05$, $**p < 0.01$. Representative results are shown on g.

Considering the anti-apoptotic role of clusterin in cancer cells, we then analyzed whether the low ability of mature CLU-KD DCs to stimulate the proliferation of allogeneic T cells might be related to a decreased survival of DCs. Indeed, assays performed at 48 hrs of culture by annexin V/propidium iodide staining revealed a marked increase in the death of LPS-treated CLU-KD DCs, but not in unstimulated CLU-KD DCs or scramble DCs either untreated or treated with LPS (Figure 5a–d). Interestingly, kinetic studies revealed that the decreased viability of LPS-treated DCs observed between days 2 and 6 is associated to a reduction in the concentration of intracellular clusterin (Figure 5e), suggesting that a reduction in cellular clusterin might be related with the decrease in DC viability observed *in vitro* after LPS stimulation. We also found that the addition of recombinant clusterin did not prevent apoptosis of CLU-KD DCs (Figure 5f), suggesting a major role for intracellular clusterin in the inhibition of LPS-induced cell death.

Taking into account the ability of clusterin to protect cells from oxidative stress in different models[37,38] and considering that the course of DC maturation is associated to the autocrine production of ROS,[39,40] we evaluated whether the anti-apoptotic effect of clusterin might be related, at least in part, to the inhibition of ROS induced DC death. In a first set of

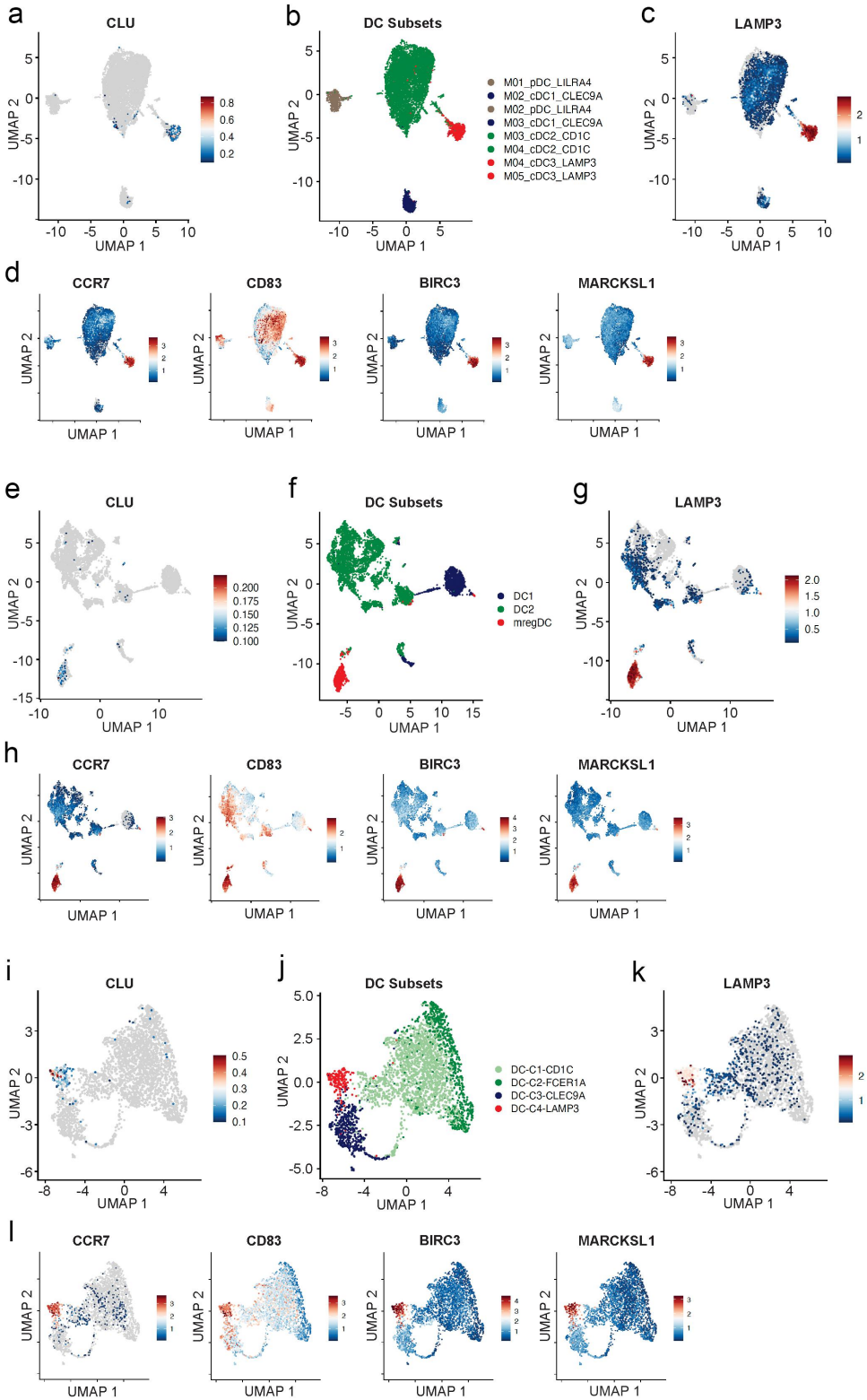

Figure 2. Clusterin is expressed by mature DCs in human cancer. a: UMAP plot showing the expression of clusterin by intratumoral DCs from the public data set published by Cheng S. et al. b: DC subsets are named as the authors did in the original publication. M04 and M05_cDC3_LAMP3 correspond to LAMP3+ DCs. The expression of the markers LAMP3, CCR7, CD83, BIRC3 and MARCKSL1 are shown in c and d. E: UMAP plot showing the expression of clusterin by intratumoral dendritic cells from a data set published by Maier et al. analyzing 35 patients with non-small cell lung cancer. f: DC subsets are named as the authors did in the original publication. The expression of the markers LAMP3, CCR7, CD83, BIRC3 and MARCKSL1 are shown in g and h. i: UMAP plot showing the expression of clusterin by intratumoral dendritic cells from a data set published by Zhang et al. analyzing 16 patients with hepatocellular carcinoma. j: DC subsets are named as the authors did in the original publication. mregDCs correspond to LAMP3+ DCs. The expression of the markers LAMP3, CCR7, CD83, BIRC3 and MARCKSL1 are shown in k and l.

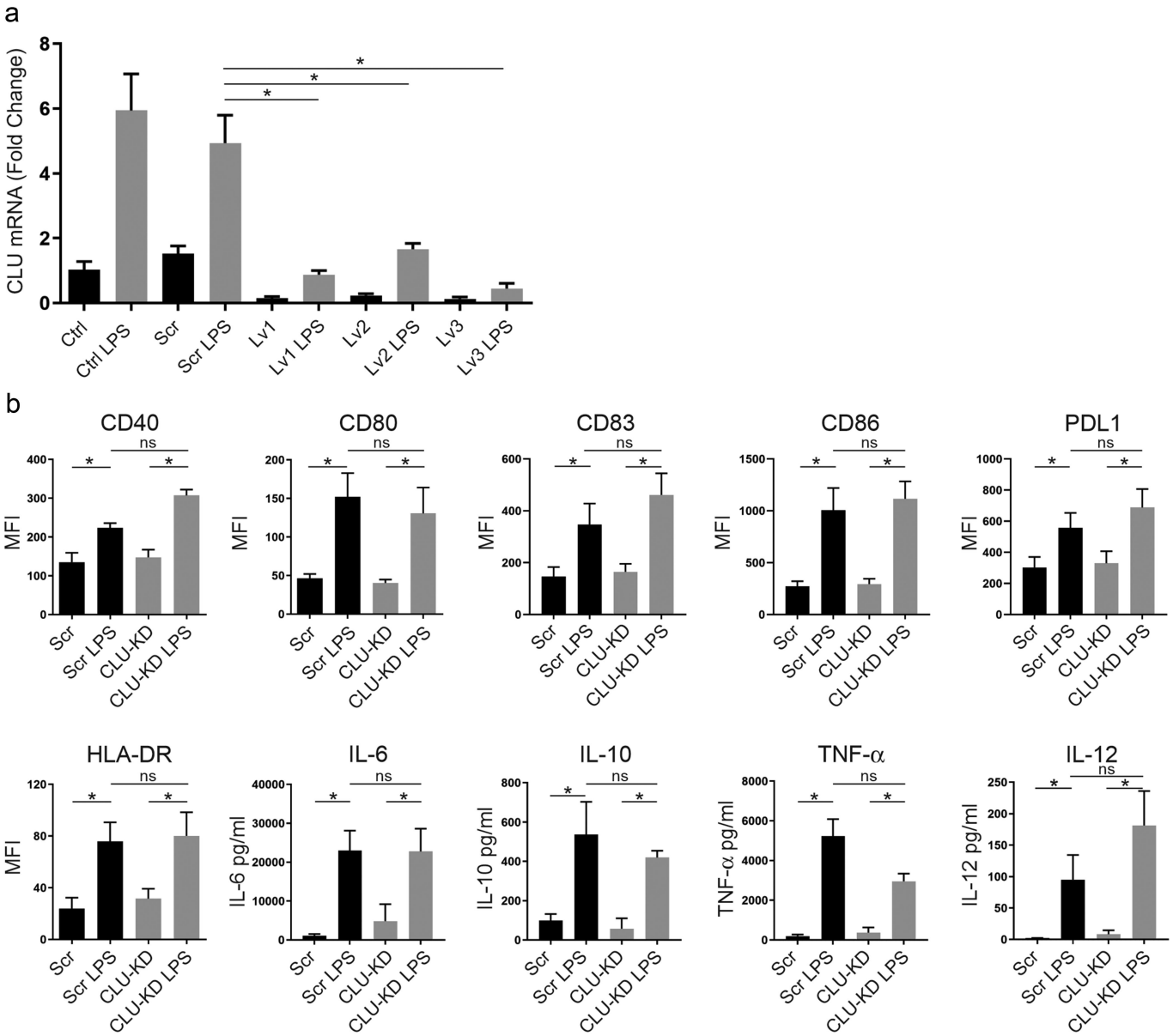

Figure 3. Analysis of maturation on CLU-KD DCs. a. MoDCs were transduced or not (Ctrl) using three different clusterin shRNA carrying lentivirus (LV1, LV2 and LV3) and a scramble lentivirus (Scr). Clusterin expression was analyzed by real time PCR after 48 hrs of LPS treatment. b: Scramble and clusterin knock-down MoDCs were incubated overnight with LPS (10 ng/ml). Cells were harvested and the expression of maturation markers (CD40, CD80, CD83, CD86, PDL1 and HLA-DR) was analyzed by flow cytometry. Cytokine production was analyzed on cell supernatants by ELISA. Data represent the arithmetic means ± the SD of 4-6 independent experiments carried out in triplicate, *=$p < 0.05$, **=$p < 0.01$, $n = 4$-6, ns=not significant. LV1 and LV3 were used in b.

experiments, we analyzed ROS production by LPS activated DCs. In agreement with previous studies,[40,41] we found that activation by LPS induced the production of mitochondrial ROS, peaking 4 hrs after stimulation in control and CLU-KD DCs (Figure 6a–d). As expected, ROS detection was inhibited by the antioxidant N-acetyl-l-cysteine (NAC) (Figure 6b,c). Interestingly, the induction of cell death mediated by LPS in CLU-KD DCs was completely inhibited by treatment with NAC (Figure 6e), suggesting that clusterin prolongs the survival of mature DC by protecting them from the oxidative stress associated to the DC maturation process. To get further insight into the inhibition of ROS-mediated cell death we decided to overexpress clusterin in LPS untreated immature MoDCs using lentiviruses carrying the clusterin gene under the *spleen focus-forming virus* (SFFV) promoter (CLU+ DCs). As expected, transduced CLU+ DCs showed a high CLU expression (Figure 6f). Scramble (Scr) and CLU+ immature DCs were exposed to ROS by treatment with tert-Butyl hydroperoxide (TBH 50–100 µM). As shown in Figure 6g,h, CLU+ DCs were shown to be resistant to TBH treatment compared with control scramble DCs. These results confirmed that clusterin expression protects DCs from ROS-mediated toxicity.

High levels of ROS production are usually found in inflamed tissues and cancer.[42,43] Our results suggest that clusterin could protect DCs from the toxic effects exerted by ROS in the tumor microenvironment. Considering that clusterin expression is not uniform in intratumoral LAMP3+ mregDCs, we decided to investigate if mregDCs enriched in clusterin expression displayed a transcriptomic profile associated with the exposure to oxidative stress. To this aim, taking advantage of the data sets

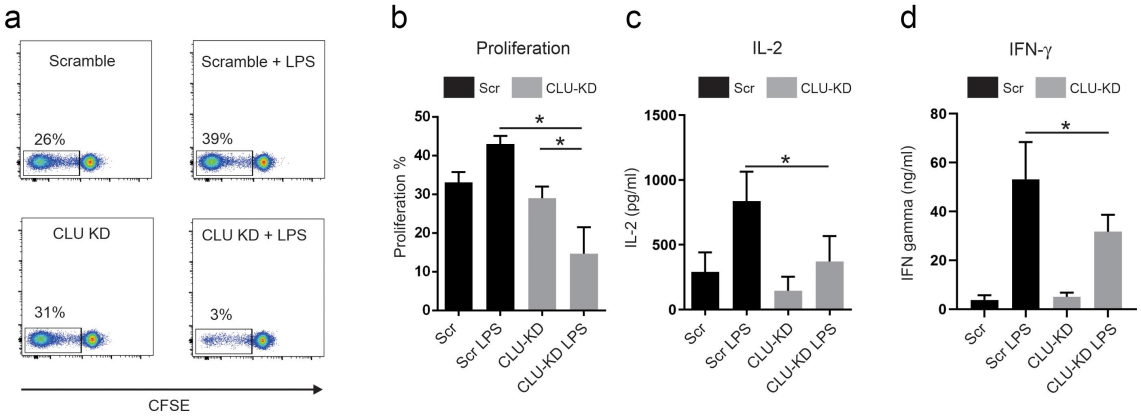

Figure 4. Clusterin expression promotes T cell priming by dendritic cells. Scramble and clusterin knock-down MoDCs were incubated or not with LPS (10 ng/ml) for 24 hs. Then, cells were co-cultured with CFSE-stained CD4+ T cells (1 DC/4 T cells ratio). After 5 days of co-culture, cell proliferation was measured by flow cytometry (a and b) and the secretion of IL-2 (c) and IFN-gamma (d) were quantified by ELISA. Data represent the arithmetic means ± the SD of 3-5 independent experiments carried out in triplicate, *=p < 0.05. LV1 and LV3 were used in all cases. A representative result is shown in a.

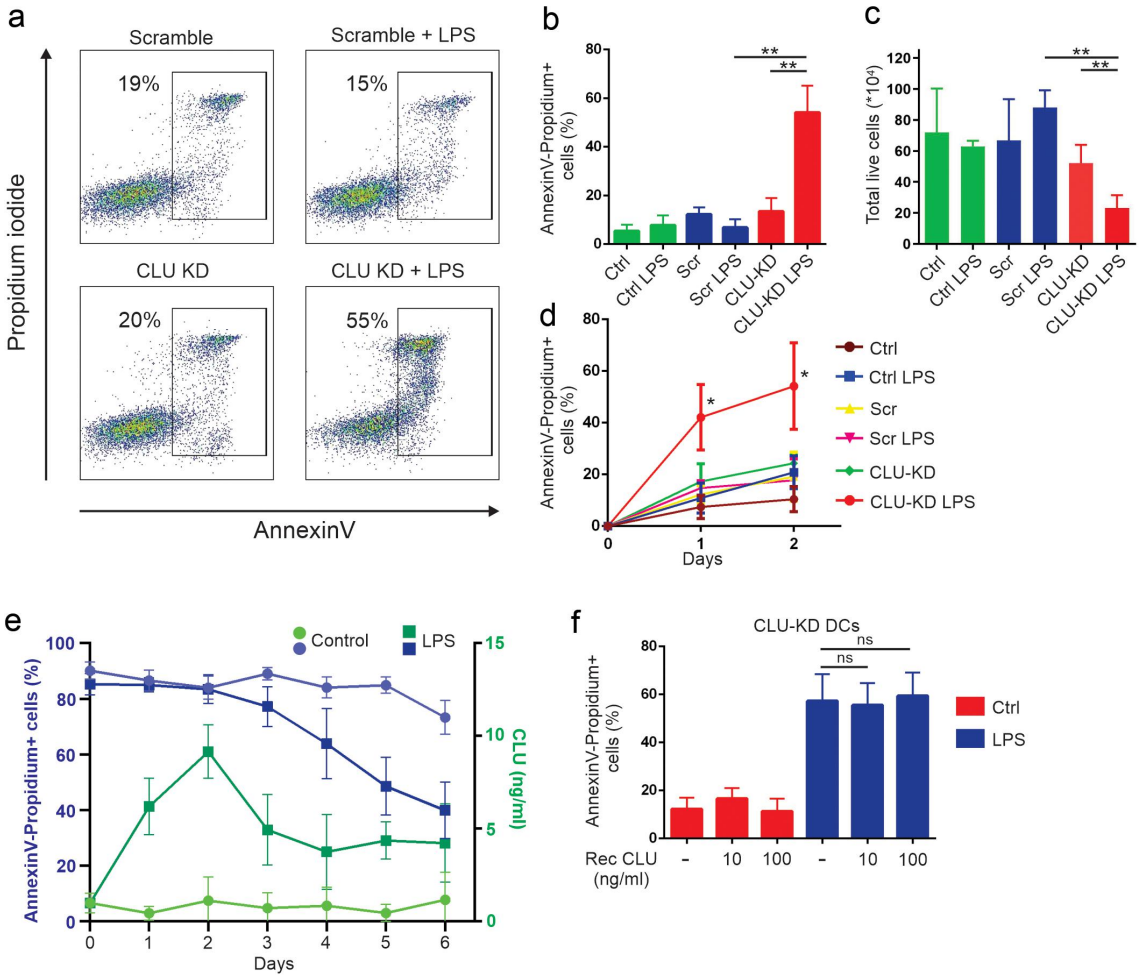

Figure 5. Clusterin inhibits mature DC death. On a-d, CLU knocked down (CLU KD), scramble and control MoDCs were treated with LPS (10 ng/ml) for 48hs and cell death was quantified by AnnexinV and propidium iodide staining by flow cytometry. e: MoDCs were treated with LPS (10 ng/ml) and cultured for 6 days. Each day, clusterin concentrations on lysed cells were measured by ELISA and DCs viability was monitored by AnnexinV and propidium iodide staining. Clusterin concentration is shown in green and cell death is shown in blue (circles=control cells, squares= LPS treated cells). f: CLU-KD DCs were treated with LPS in the presence or absence of recombinant clusterin (10 or 100 ng/ml) for 48 hs. The cells were stained using AnnexinV and propidium iodide and analyzed by flow cytometry. Data represent the arithmetic means ± the SD of 3-6 independent experiments carried out in triplicate, **=p < 0.01. LV1 and LV3 were used in all cases. Representative experiments are shown in a and e.

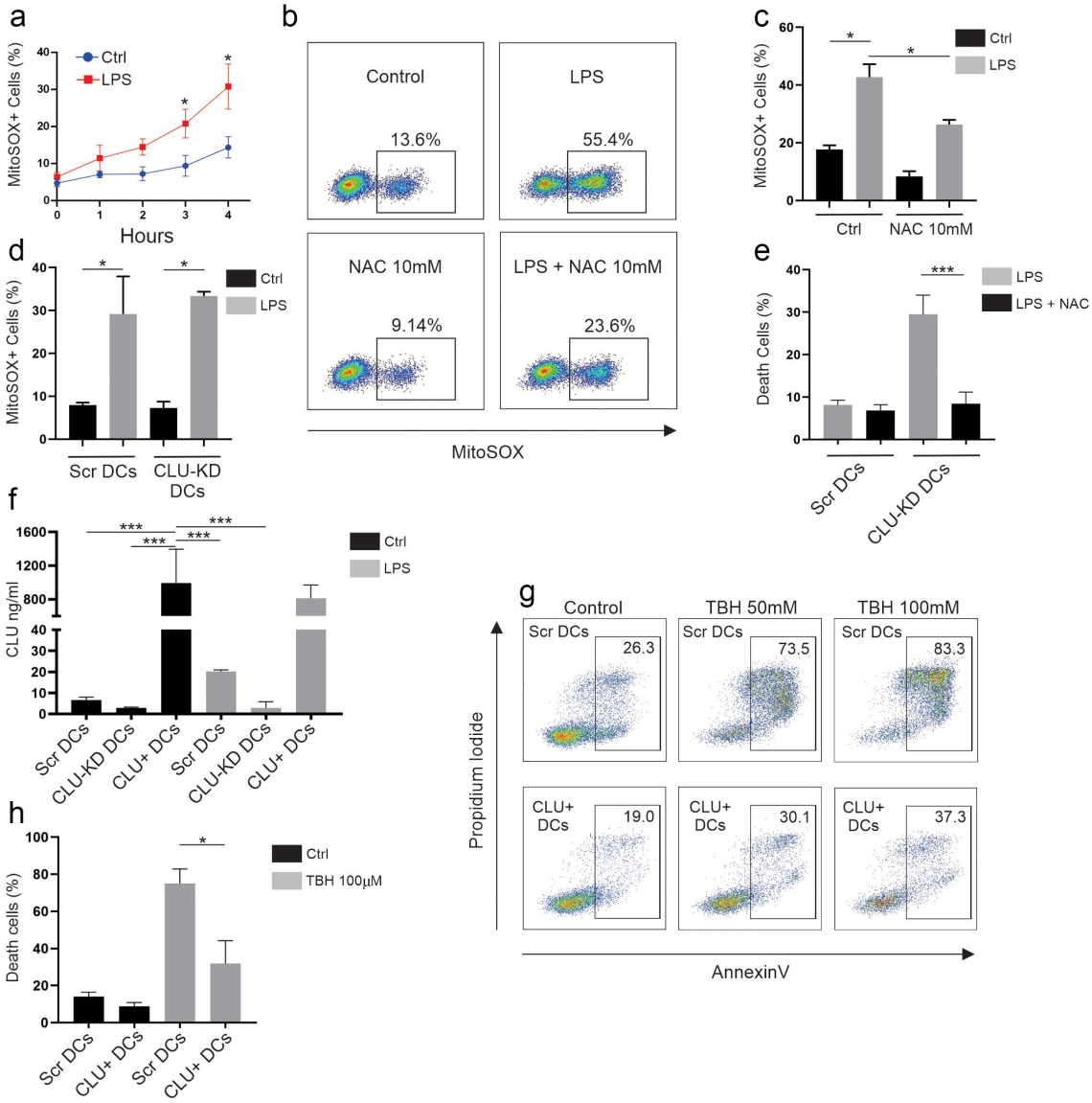

Figure 6. Clusterin protects DCs from ROS induced cell death. a-c: MoDCs were treated with LPS (10 ng/ml) and/or N-Acetyl Cysteine (NAC, 10 mM) for 1-4 hs, stained using MitoSOX and analyzed by flow cytometry (4 hrs treatment on b and c). d: scramble and clusterin knock-down (CLU-KD) MoDCs were incubated or not with LPS (10 ng/ml) for 4 hs, stained using MitoSOX and analyzed by flow cytometry. On e, scramble and clusterin knock-down (CLU-KD) MoDCs were incubated or not with LPS (10 ng/ml) and/or N-Acetyl Cysteine (NAC, 10 mM) and cell death was analyzed after 48 hrs by AnnexinV and propidium iodide staining and flow cytometry. f: MoDCs were transduced using lentiviruses carrying the CLU gene under the SFFV promoter (CLU+ DCs). After 48hs clusterin concentration was measured by ELISA on cell lysates of scramble DCs (Scr), CLU knock-down DCs (CLU-KD) and CLU+ DCs (treated or not with LPS). On g and h, scramble (Scr) and CLU+ DCs were treated with tert-Butyl hydroperoxide (TBH 50-100 µM) and cell death was evaluated by AnnexinV and propidium iodide staining and flow cytometry. Data represent the arithmetic means ± the SD of 3-5 independent experiments carried out in triplicate, *=$p < 0.05$, ***=$p < 0.001$. LV1 and LV3 were used in all cases. Representative experiments are shown in b and g.

previously analyzed in Figure 2, we selected the genes with an expression positively or negatively correlated with the expression of clusterin in the entire population of mregDCs (spearman correlation coefficient > 0.5 or <-0.5 in at least two data sets) (Suppl table S1 and S2) and performed a gene set enrichment analysis (GSEA) using Gene Ontology (Figure 7a,b). Clusterin expression was shown to be associated with processes related with response to stress, metabolic adaptations and response to cell damage (Figure 7a). The expression of clusterin also correlated with gene signatures related with response to oxidative stress and cell damage, such as p53 signaling pathway (Figure 7c). These results suggest that intratumoral LAMP3+ mregDCs with high clusterin expression use different gene programs to deal with environmental stressful conditions. Of note, clusterin expression is not associated with cell death pathways in GSEA or apoptotic signatures (Suppl Figure S3), suggesting that high clusterin expressing LAMP3+ mregDCs are successful in their attempts to survive. On the other side, mregDCs with low clusterin expression are highly expressing genes related with active immune functions, antigen presentation and myeloid activation (Figure 7b,c).

Discussion

The survival of DCs is regulated by the multiple interactions that these cells establish with their environments. In mice models, it has been proposed that different maturation stimuli might activate a molecular "timer" that regulates the survival

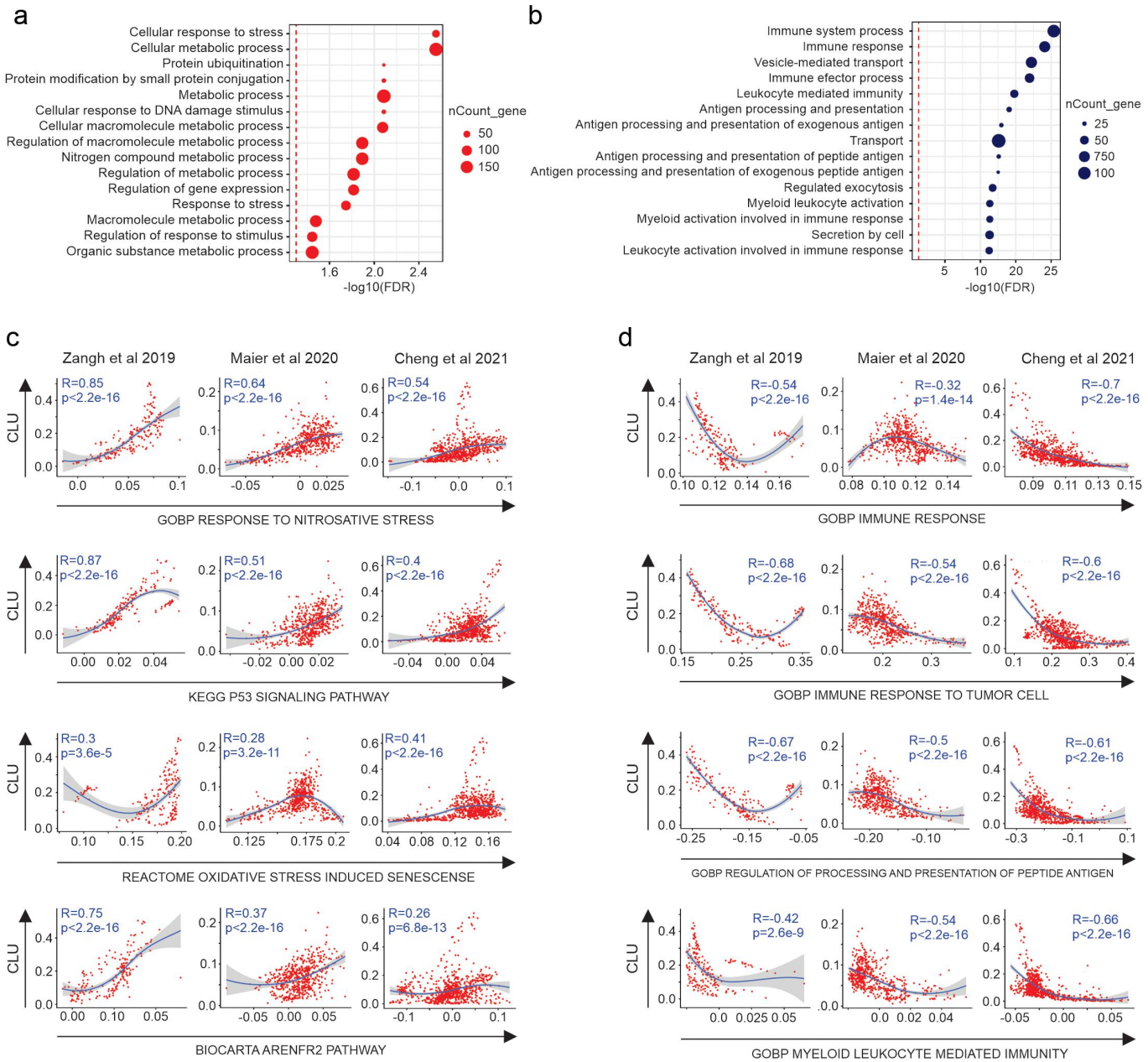

Figure 7. High clusterin expression correlates with a stressed DC phenotype. a and b: Top 15 enriched Gene Ontology terms of genes with positive (a) and negative (b) correlated expression with clusterin (spearman correlation coefficient > 0.5 or <-0.5 in at least two data sets) on LAMP3+ mregDCs. C and D: Positive (c) and negative (d) correlations between clusterin expression and gene signatures on LAMP3+ mregDCs on the three data sets analyzed.

and consequently the immunogenicity of DCs.[44] The stimulation of murine DCs with LPS induces cell death after a period of 4–5 days, an appropriate time frame to ensure T cell priming but preventing, at the same time, an excessive T cell stimulation and the risk of autoimmunity.[44] Indeed, the lifespan of DCs seems to be critical for the regulation of adaptive immune response. While a defective DC apoptosis results in a sustained lymphocyte activation and systemic autoimmune manifestations, an increased DC death was shown to be associated with immunosuppression in different pathological conditions.[7,45] The mechanisms that regulate cell death in human DCs have been poorly studied.[6,7,9,10] Using MoDCs as a model, we here show that human DC activation by LPS triggers the production of ROS which are able to promote cell death but, at the same time, the activation of MoDCs leads to the up regulation of clusterin expression preventing the deleterious action of ROS. Of note, after LPS stimulation, DCs produce not only mitochondrial ROS but also high concentrations of phagosome ROS, that might also compromise cell viability.[46] In this scenario, we speculate that the expression of clusterin may be important to gain time for an efficient T cell activation.

Production of high levels of ROS, originated from cancer cells and infiltrating immune cells, is a hallmark of cancer progression and resistance to treatment.[42,47] Reactive oxygen species promote carcinogenesis and tumor growth, and also modulate the function of different cell types in the tumor microenvironment.[43] However, high ROS concentrations induce cell death. Although the role of clusterin in promoting

tumor cell survival has been studied in detail, it has been poorly explored in non-neoplastic cells including leukocytes.[48,49] In line with the *in vitro* results presented here, we have found that clusterin is selectively expressed in the human tumor microenvironment in a population of mature LAMP3 expressing DCs called mregDCs, but not in immature DCs. This DC state is widely present in different tumor types and probably arises from the activation of cDC1 and cDC2 in the tumor microenvironment.[32,33] mregDCs display an immunoregulatory program associated with the capture of cancer cell-associated antigens.[33] A recent report suggest that these cells are critical for the differentiation of progenitor CD8+ T cells into effective antitumor CD8+T cells upon checkpoint blockade immunotherapy.[50] We speculate that, as we observed *in vitro*, inside tumors clusterin might be up-regulated upon DC activation to promote the survival of mature DCs. Interestingly, we found that high clusterin expression in mregDCs correlates with the activation of gene programs mainly related to cell response to stress, suggesting that clusterin might play a role in promoting DC survival under the stressing conditions of tumor microenvironments.

Dendritic cells play a critical role in the initiation of antigen-specific anti-tumor immunity and tolerance, by sampling and presenting antigens to T cells.[32] In order to acquire antigens for presentation to T cells, DCs must establish close contact with dying cancer cells, which are the main source of tumoral antigens.[51] At the same time, necrotic cells release different factors able to induce DC maturation. We have shown here that different conditions found in the tumor microenvironment, such as necrotic cells, HMGB1, acidic pH and TNF-α induce the expression of clusterin by MoDCs. We speculate that, in the tumor microenvironment, clusterin expression might be induced by these factors together with DC activation to promote the survival of mature DCs. Our results suggest a paradoxical effect of clusterin on the tumor microenvironment. Indeed, while clusterin expression by cancer cells promote tumor growth and resistance to therapy,[12,52] the expression of clusterin by intratumoral DCs might favor the anti-tumor immune response.

We also found that clusterin expression is induced after LPS stimulation not only in DCs, but also in macrophages and monocytes. Interestingly, in contrast with DCs, clusterin knock down in macrophages didn´t compromise cell viability (unpublished results) suggesting a different role for clusterin in both cell types. Further studies are needed to characterize the role of clusterin in macrophages and monocytes.

One of the limitations in our study is the utilization of moDCs as a model of human DCs. Indeed, moDCs does not faithfully represents human DC populations in vivo.[53] Moreover, using this model, each independent experiment involves the use of a different blood donor, that explain differences among experiments in terms of variability. However, we consider that MoDCs represent the best available in vitro DC model to perform gene knockdown experiments. To confirm our observations in other DCs populations, the expression of clusterin in mature DCs was also demonstrated using isolated blood BDCA+ DCs and public data sets of cancer and tonsil mature human DCs. Another limitation of the model is the scarce amounts of cells available for the experiments after DC transduction. For this reason, we were unable to screen a variety of cell death pathways different from those induced by ROS.

In neoplastic cells, clusterin expression is associated with greater aggressiveness, worse prognosis, and resistance to chemotherapy and radiotherapy.[54–59] Different mechanisms have been described by which clusterin could promote tumor growth, among these, the best characterized is the inhibition of apoptosis of neoplastic cells. However, contradictory results have been reported in colon cancer, breast cancer and prostate cancer.[56,58,60] The role of clusterin expression in cancer progression remains to be clearly defined. Our observations suggest that clusterin might promote the survival of mature DCs under the stressing conditions of tumor microenvironments.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All relevant data is contained within the article: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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