Bourazopoulou E, Kapsogeorgou EK, **Routsias JG**, Manoussakis MN, Moutsopoulos HM, Tzioufas AG. **Λειτουργική έκφραση του υποδοχέα της α2-μακροσφαιρίνης, CD91, στα επιθηλιακά κύτταρα των σιελογόνων αδένων.** J Autoimmun. 2009, 33(2):141-6.

CD91 molecule is a multifunctional receptor of alpha 2-macroglobulin, heat-shock proteins and calreticulin. CD91 has been implicated in cross-presentation of peptides chaperoned by these proteins to MHC molecules, thus eliciting antigen-specific immune responses. Hence, CD91 is considered as a major regulator of innate and acquired immune responses. Herein, we show that CD91 molecules are expressed by human salivary gland epithelial cells (SGEC), as indicated by immunohistochemical studies in minor salivary gland biopsy tissues (n = 21) as well as by the analyses of human long-term cultured non-neoplastic SGEC lines (n = 11) and the neoplastic HSG cell line. In these cell lines CD91 expression was evaluated by RT-PCR, flow cytometry and confocal microscopy. Standard internalization assays revealed that HSG and SGECs are capable to bind and internalize the CD91 ligand alpha 2-macroglobulin. This internalization is specific, as attested by inhibition studies using unlabeled alpha 2-macroglobulin and a blocking antibody against human CD91 receptor. Conclusively, our findings indicate that SGEC functionally express CD91 receptor, suggesting that this pathway might be involved in the presentation of exogenous antigens in SGEC.

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Functional expression of the alpha 2-macroglobulin receptor CD91 in salivary gland epithelial cells

Efi Bourazopoulou, Efstathia K. Kapsogeorgou, John G. Routsias, Menelaos N. Manoussakis, Haralampos M. Moutsopoulos, Athanasios G. Tzioufas*

Department of Pathophysiology, School of Medicine, National University of Athens, 75 Mikras Asias Street, 11527 Athens, Greece

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ABSTRACT

CD91 molecule is a multifunctional receptor of alpha 2-macroglobulin, heat-shock proteins and calreticulin. CD91 has been implicated in cross-presentation of peptides chaperoned by these proteins to MHC molecules, thus eliciting antigen-specific immune responses. Hence, CD91 is considered as a major regulator of innate and acquired immune responses. Herein, we show that CD91 molecules are expressed by human salivary gland epithelial cells (SGEC), as indicated by immunohistochemical studies in minor salivary gland biopsy tissues ($n = 21$) as well as by the analyses of human long-term cultured non-neoplastic SGEC lines ($n = 11$) and the neoplastic HSG cell line. In these cell lines CD91 expression was evaluated by RT-PCR, flow cytometry and confocal microscopy. Standard internalization assays revealed that HSG and SGECs are capable to bind and internalize the CD91 ligand alpha 2-macroglobulin. This internalization is specific, as attested by inhibition studies using unlabeled alpha 2-macroglobulin and a blocking antibody against human CD91 receptor. Conclusively, our findings indicate that SGEC functionally express CD91 receptor, suggesting that this pathway might be involved in the presentation of exogenous antigens in SGEC.

1. Introduction

CD91 is a multifunctional receptor that binds and internalizes numerous, structurally diverse ligands, thus participating in a broad spectrum of physiological cellular processes [1–5]. Initially, CD91 was identified as a member of the low-density lipoprotein receptor family (low-density lipoprotein receptor-related protein, LRP1) possessing an active role in lipid metabolism, and subsequently as a receptor of activated alpha 2-macroglobulin (alpha 2-macroglobulin receptor, A2MR) [1,2,6]. During the last decade, CD91 was recognized as a major sensor of innate immunity. It is the common receptor that in classical antigen-presenting cells mediates the internalization of heat-shock proteins (such as gp96, hsp70 and hsp90) and calreticulin leading to the cross-presentation of the peptides carried by these proteins and thus, eliciting specific innate and adaptive immune responses against these peptides [6–10]. The interaction of CD91 with the chaperone protein calreticulin is of special interest, since it is the main pathway for clearance of apoptotic cells [8,11–13].

Due to its diverse roles, CD91 expression and distinct functions have been investigated in a variety of cell types. The expression of CD91 receptor in human and mouse monocytes and dendritic cells and its key role in the phagocytosis of apoptotic cells and cross-presentation of antigenic peptides is well-established [6–12], whereas CD91 receptor has been shown to participate in the phagocytic capacity of mouse neoplastic mammary epithelial cells [14]. Furthermore, CD91 receptor has been implicated in the migration/invasion of human keratinocytes and retinal pigment cells [15,16].

Emerging evidence supports the notion that human salivary gland epithelial cells (SGECs) are key regulators of the local immune responses in patients with Sjögren's syndrome (SS). Over the last decade studies have shown that long-term cultured SGECs constitutively express several immunoreactive molecules that are implicated in antigen-presentation, lymphoid cell recruitment and activation, and innate responses [17–20]. Due to the foremost role of CD91 in the trafficking of innate receptors and the presentation of their chaperoned peptides, we sought to study its expression by human salivary gland epithelial cells, including the neoplastic HSG cell line, as well as long-term cultured non-neoplastic SGECs. In addition, the in-situ expression of CD91 receptor by SGEC was examined in minor salivary gland (MSG) biopsy samples from SS patients and controls. Furthermore, the function of CD91 molecules was investigated by the cell binding and internalization of methylamine-activated FITC-labeled alpha 2-macroglobulin, a well known ligand of CD91.

* Corresponding author. Tel.: +30 210 7462670; fax: +30 210 7462664.
E-mail address: agtzi@med.uoa.gr (A.G. Tzioufas).

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2. Materials and methods

2.1. Reagents

Unconjugated or fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against human CD91 (clone A2Mr alpha-2) were purchased from Serotec (Oxford, UK) and the isotype-matched control MoAb was purchased from BD-Biosciences (San Jose, CA, USA). Unlabeled and FITC-labeled methylamine-activated alpha 2-macroglobulin (alpha 2-macroglobulin-MA) was obtained from BioMac (Leipzig, Germany).

2.2. Cell cultures and biopsy specimens

Non-neoplastic, long-term cultured SGEC lines ($n = 11$) were established from minor salivary gland (MSG) biopsy samples obtained from patients undergoing diagnostic evaluation, as previously described [21]. Six of these SGEC lines were established from patients that were diagnosed as SS according to the American–European classification criteria [22], whereas five from sicca-complaining individuals that did not fulfill the above-mentioned criteria and did not exhibit histopathologic or serological features consistent with SS (referred hereafter as controls). Informed consent from all participants and regional hospital ethical committee approval were received for this study. The purity and epithelial origin of cultured SGEC lines was routinely verified by morphology, the uniform expression of epithelial-specific markers and the absence of markers indicative of lymphoid/monocytoid cells [21]. Distinct SGEC lines have not been found to differ in morphological features, proliferation rate or survival [21].

The human salivary gland (HSG) cell line was purchased from European Collection of Cell Cultures (ECACC No. 95031024, Salisbury, UK) and was maintained in MEM medium supplemented with 1% NEAA, 10% fetal bovine serum (FBS), L-glutamine (2 μM), penicillin (50 U/ml), and streptomycin (50 mg/ml) (all purchased from Invitrogen, Carlsbad, CA, USA).

Peripheral blood mononuclear cells (PBMC) and peripheral blood monocytes (analyzed by differential gating of PBMCs based on appropriate forward and side scatter parameters, as well as in positive staining for CD14 molecule) were used as a positive control population for CD91 expression. PBMC were separated from healthy donors ($n = 3$) by density-gradient centrifugation on Ficoll/Paque (Amersham-Pharmacia Biotech, Uppsala, Sweden).

Labial MSG biopsy tissues obtained from twelve SS patients and nine individuals complaining for dry mouth, but with negative MSG biopsy, were studied immunohistochemically for CD91 expression. Subcutaneous abdominal white adipose and tonsil tissues were obtained after informed consent from patients undergoing abdominal surgery and tonsillectomy, respectively, at the Department of Surgery of Laiko Hospital (Athens, Greece) and served as positive control tissues.

2.3. Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA, USA). 0.5 μg RNA was reversed transcribed using oligo(dT) primers (Eurofins-MWG-Operon; Ebersberg, Germany) and the Superscript-II reverse transcriptase (Invitrogen). The specific primer sets (Eurofins-MWG-Operon) used for the amplification of CD91 were: forward: 5′-CACCTTAACGGGAGCAATGT-3′, reverse: 5′-GTTTCCCAGTCGGTCCAGTA-3′ (producing a 494-bp amplicon). The integrity of all cDNA samples was tested by RT-PCR for beta-actin mRNA using the following primers: forward: 5′-TTCTACAATGAGCTGCGTGTG-3′, reverse: 5′-CGATGCCAGTGGTACGG-3′, (187-bp product). The PCR included an initial denaturation step at 95 °C for 5 min, 33 cycles of 95 °C for 45-sec, 57 °C for 1-min and 72 °C for 1-min followed by a final extension step at 72 °C for 10-min. The amplified fragments were size-separated in 1.5% agarose gel containing ethidium bromide and visualized on a Vilber Lourmat ultraviolet photo-system (Marne La Valle'e, France). The amplified products were isolated by the QIAquick gel extraction kit (Qiagen) and identified by automated sequencing (Eurofins-MWG-Operon).

2.4. Flow cytometry

To analyze surface CD91 expression at least 5×10^4 cells were stained with FITC-conjugated anti-CD91 or isotype-matched control mAbs, as previously described [19]. For analysis of the intracellular expression, prior to staining, the cell membranes were permeabilized by FACS permeabilizing solution (BD-Biosciences), according to manufacturer's instructions. In certain experiments, the capacity of CD91 receptor that is expressed by epithelial cells to bind its main ligand, alpha 2-macroglobulin, was assessed by staining with FITC-labeled alpha 2-macroglobulin-MA (100 μg/ml). The specificity of reaction was confirmed by inhibition with unlabeled methylamine-activated alpha 2-macroglobulin (20-fold excess) or anti-CD91 (2-fold excess) antibody and by negative staining with an FITC-labeled irrelevant protein (human ovalbumin). Analyses were performed using the FACSCalibur flow cytometer and CellQuest software (BD-Biosciences). Mean fluorescence intensity values obtained by staining with specific mAbs were corrected by the subtraction of background values (isotype-control mAbs) [19].

2.5. Confocal microscopy

The analysis of CD91 expression by epithelial cells using confocal microscopy was performed in cells grown in 16-well chamber slides (Nunc Inc., Napierville, IL) by standard technique. Briefly, cells were fixed with methanol for 10 min followed by acetone for 2 min at −20 °C. Nonspecific antibody binding was blocked by incubation with 2% non-immune fetal bovine serum. Staining was performed by overnight incubation with FITC-conjugated anti-human CD91 or isotype-matched control antibodies at 4 °C in a humidified chamber. Unstained cells were routinely used in confocal analyses for the standardization of the excitation/emission filter sets in order to exclude cell autofluorescence. Images were acquired by a Leica TCS-SP5 confocal microscope.

2.6. Immunohistochemical analysis

The expression of CD91 was analyzed in formalin-fixed paraffin-embedded MSG biopsy tissue sections (5 μm) by a standard immunoperoxidase technique using the EnVision system (Dako, Glostrup, Denmark). Antigen retrieval was performed by microwaving in 10 mM citrate buffer (pH 6.0). Non-immune fetal bovine serum (2%) and 0.5% H_2O_2 in methanol were used to block nonspecific antibody binding and endogenous peroxidase activity, respectively. Negative-control staining was performed by replacing primary with irrelevant isotype-matched antibodies. Biopsy sections were counterstained with hematoxylin, dehydrated and mounted in DPX (BDH, Chemicals, Poole, UK).

2.7. Uptake of FITC-labeled alpha 2-macroglobulin-MA

A standard internalization assay was employed to assess the functionality of CD91 receptor. This method enables the binding of the labeled ligand at 4 °C and the monitoring of its internalization for increasing time intervals at 37 °C. Briefly, cells were grown in

16-well chamber slides. At confluence, cells were washed twice with serum-free KBM culture medium and incubated with 100 μg/mL of FITC-labeled alpha 2-macroglobulin-MA or FITC-labeled irrelevant protein (human ovalbumin, which served as negative control) in serum-free KBM culture medium for 30 min at 4 °C, followed by incubation for different time points (0, 5, 10, 15 min) at 37 °C. Subsequently, cells were washed with phosphate-buffered saline, fixed with methanol for 10 min at −20 °C and mounted in an anti-fading mounting medium (INOVA Diagnostics, San Diego, CA, USA). For inhibition experiments, cells were either incubated with 100 μg/mL of anti-human CD91 monoclonal antibody for 1 h prior to the addition of FITC-labeled alpha 2-macroglobulin-MA or with a mixture of labeled and unlabeled alpha 2-macroglobulin-MA in a molar ratio of 1:10 for competitive assessment of ligand internalization. The alpha 2-macroglobulin ligand uptake was documented by confocal microscopy analysis.

3. Results

3.1. Expression of CD91 receptor in salivary gland epithelial cells

The expression of CD91 mRNA was readily detected in the neoplastic HSG epithelial cell line, as well as in all the long-term cultured non-neoplastic SGEC lines ($n = 6$) examined, as indicated by the amplification of a product of the expected molecular size (494 bp) in all the cell lines tested using a specific RT-PCR (Fig. 1A). Sequencing analysis confirmed that the product corresponded to CD91 mRNA (data not shown). Flow cytometric analysis indicated low, but definite, constitutive surface expression of CD91 protein by HSG and SGEC ($n = 11$) (Fig. 1B), whereas peripheral blood monocytes exhibited high surface CD91 staining (Fig. 1B). Intracellular staining revealed significant constitutive intracellular expression of CD91 receptor in all the cell lines tested (Fig. 1B). No differences were observed in the expression of CD91 by SGEC lines obtained from patients with primary SS ($n = 6$) or control individuals ($n = 5$) (data not shown). Analysis of protein expression by confocal microscopy further confirmed the expression of CD91 receptor by HSG and SGEC cell lines (Fig. 1C). Treatment of salivary gland epithelial cells with IFNγ did not have any effect on the constitutive expression of CD91 (data not shown).

Subsequently, we examined whether epithelial CD91 expression occurs in-situ in MSG tissues. The immunohistochemical analysis revealed definite expression of CD91 receptor in ductal, but not in acinar, epithelial cells of salivary glands (Fig. 2). Similar expression pattern by the epithelia was detected in MSG tissues obtained from pSS patients or controls (Fig. 2). The mononuclear cells that

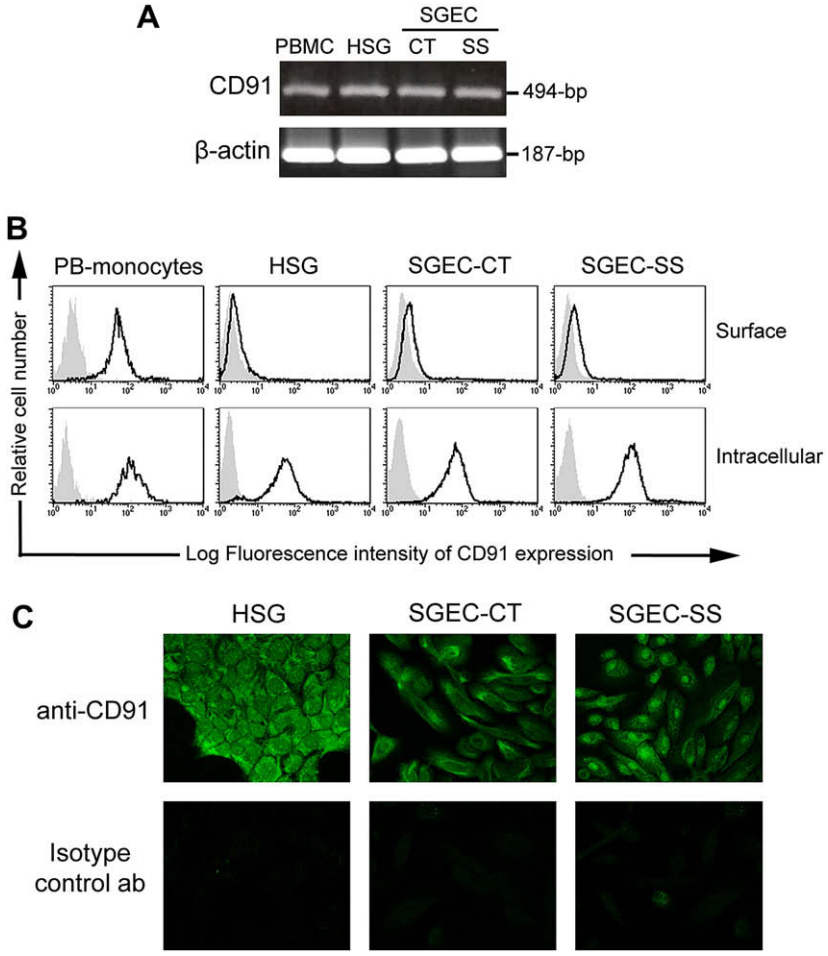

Fig. 1. CD91 receptor is expressed by salivary gland epithelial cells. A. Representative example of agarose gel electrophoresis showing the amplification of CD91 mRNA by RT-PCR in human peripheral blood mononuclear cells (PBMC), HSG cell line and SGECs established from a control individual (CT) and an SS patient (SS). The amplification of β-actin mRNA served as a control for the evaluation of cDNA integrity. B. Representative example of surface and intracellular expression of CD91 protein by peripheral blood (PB) monocytes, HSG cells and SGECs derived from a control individual (CT) and an SS patient (SS), as assessed by flow cytometry using a FITC-conjugated anti-CD91 mAb (black line). The light grey-filled histogram represents the staining with the isotype-control antibody. C. Representative example of expression of the CD91 receptor by the HSG cell line and SGEC lines obtained from a control individual (CT) and an SS patient (SS), as assessed by confocal microscopy using a FITC-conjugated anti-CD91 mAb. Staining with an isotype-control antibody, which results to absence of fluorescence signal, is also shown (lower panel). (Original magnification: × 400).

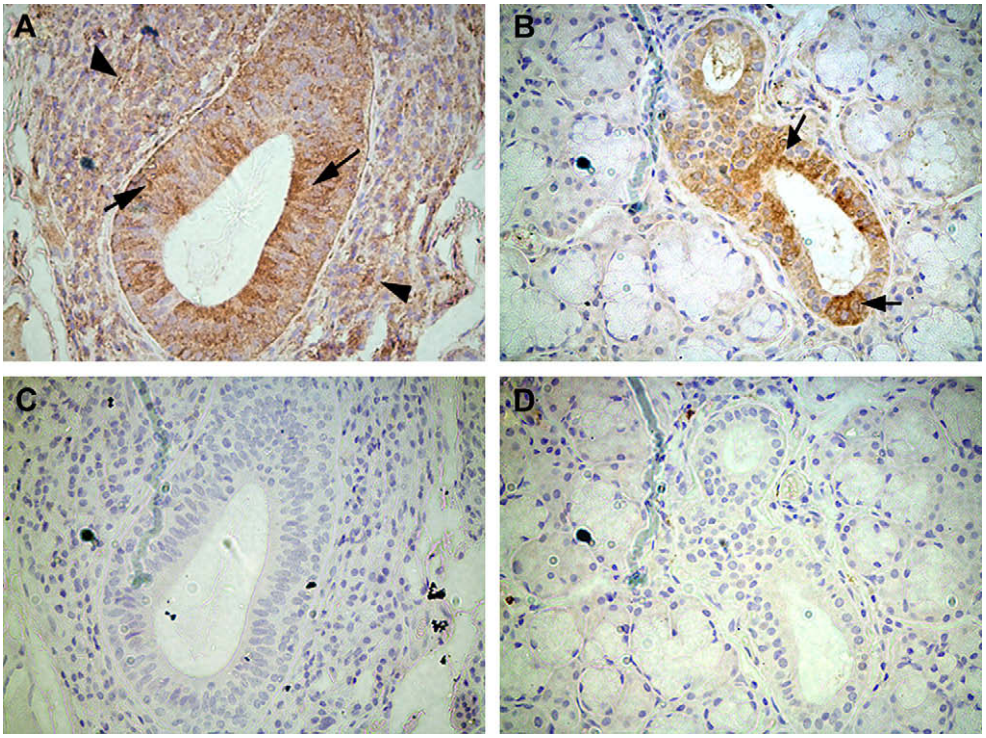

Fig. 2. Representative example of immunohistochemical detection of CD91 receptor expression at minor salivary gland (MSG) tissues obtained from SS patients (A) and control individuals (B). Positive CD91 staining is detected on ductal salivary gland epithelial cells of SS and controls (indicated by arrows), as well as on the mononuclear cells (arrowheads) that infiltrate the MSG tissues of SS patients. No differences were observed in the expression of CD91 by ductal epithelia in SS and controls. Absence of staining of MSG tissues with an isotype-matched control antibody (C, D) (Original magnification x 400).

infiltrate the inflamed MSG tissues of SS patients were also positive for CD91 staining. Adipose and tonsil tissues were used as control tissues for antibody optimization (data not shown).

3.2. Salivary gland epithelial cells express a functional CD91 receptor

To investigate the ability of epithelial CD91 receptor to function and mediate cell-endocytosis, we evaluated using two methods the capacity of salivary gland epithelial cells to bind and internalize one of the major CD91 ligands, the alpha 2-macroglobulin. Flow cytometric analysis revealed that FITC-labeled alpha 2-macroglobulin binds to CD91-expressing HSG and SGEC cells (Fig. 3A), whereas FITC-labeled human ovalbumin does not (data not shown). This binding was specific and was mediated by the CD91 receptor, since it was inhibited by an inhibitory anti-CD91 mAb or unlabeled alpha 2-macroglobulin (Fig. 3A). Approximately 29% inhibition of alpha 2-macroglobulin staining was achieved by both blocking reagents. Furthermore, a standard internalization assay further confirmed that CD91 receptor expressed by HSG and SGEC cells is functional and capable of mediating an endocytic pathway of ligand internalization (Fig. 3B). Thus, confocal microscopy analysis of HSG and SGEC cells revealed that FITC-labeled alpha 2-macroglobulin localized at the cellular membrane at 0 min and 5 min. However, a punctuate fluorescence pattern, which indicated that CD91 ligand was distributed into discrete vesicular compartments of the cytoplasm, was detected at 10 and 15 min (Fig. 3B). This procedure was specific, since it was completely inhibited by the pre-treatment of cells with either an inhibitory monoclonal antibody against CD91 or by an excess fold of unlabeled alpha 2-macroglobulin (Fig. 3B), but not by an isotype matched monoclonal antibody or FITC-labeled human ovalbumin (data not shown). Furthermore, in parallel experiments FITC-labeled human ovalbumin was not found able to bind on SGEC or HSG cells (data not shown).

It is not clear why the alpha 2-macroglobulin surface staining of epithelial cells is only partially inhibited by blocking agents, whereas its internalization by the same cells is completely inhibited. Binding of alpha 2-macroglobulin in additional surface receptors that do not mediate its internalization and/or high trafficking rates of surface CD91 receptor might participate. No differences were observed in the internalization of alpha 2-macroglobulin by SGEC lines obtained from SS patients or control individuals.

4. Discussion

In this study, we show for the first time that the CD91 receptor is constitutively expressed in human salivary gland epithelial cells. Despite the low constitutive surface expression of CD91 receptor in salivary gland epithelial cells, it was able to specifically mediate the internalization of its ligands. The similar pattern of CD91 expression by ductal epithelial cells in inflamed or non-inflamed MSG tissues suggests that its expression is not modulated by the inflammatory microenvironment. The regulation of CD91 expression in other inflammatory disorders remains elusive. Recent findings have shown high membrane CD91 expression on synovial tissue in rheumatoid arthritis [23], whereas it has been reported that $CD91^{+}$-dendritic cells juxtaposed to keratinocytes expressing excess heat-shock proteins (HSPs) in psoriatic plaques [24]. In addition, up-regulated monocytic CD91 expression has been correlated with slow progression in melanoma patients [25], whereas low CD91 expression has been reported in long-term non-progressing HIV-1-infected patients [26].

CD91 receptor and its interaction with cell-products of apoptotic or necrotic cells, such as heat-shock proteins and calreticulin, place

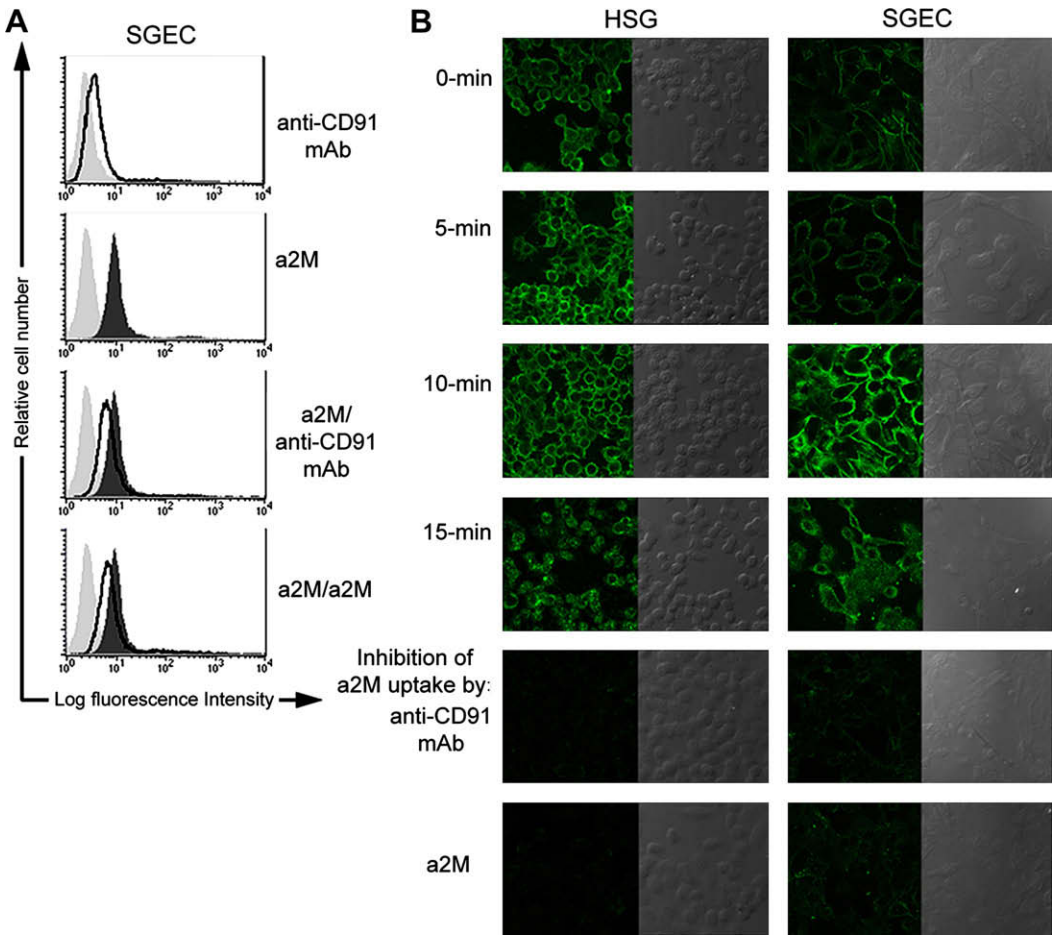

Fig. 3. The CD91 molecules that are expressed by salivary gland epithelial cells bind and internalize FITC-conjugated alpha 2-macroglobulin. A. Flow cytometric analysis shows that CD91-expressing SGECs (first histogram; CD91 staining is displayed by the black-line) are stained with FITC-conjugated alpha 2-macroglobulin (α2M; second histogram). The α2M-staining of epithelial cells is inhibited by pre-incubation with either an inhibitory anti-CD91 antibody (α2M/anti-CD91 mAb; third histogram) or unlabeled α2M (α2M/α2M; fourth histogram). In the second to fourth panels the dark grey-filled histograms represent the staining with FITC-conjugated α2M. The black-lined histogram in the third and fourth panel corresponds to staining with FITC-conjugated α2M after inhibition with either an inhibitory anti-CD91 mAb or unlabeled α2M, respectively. Staining with an isotype-control antibody is also shown (light grey-filled histograms). A representative of two experiments is shown. B. Representative example of confocal microscopy analysis of the FITC-conjugated alpha 2-macroglobulin uptake by HSG or SGEC cell lines in different time intervals (0, 5, 10, 15 min). Cells were pre-incubated with FITC-conjugated alpha 2-macroglobulin (α2M) to enable binding of α2M on the cell surface for 30 min at 4 °C (shown at 0 min). The cell-surface bonded α2M is internalized at the next time points leading to a punctuate cytoplasmic staining of epithelial cells at 15-min. The radical loss of the cytoplasmic staining that is observed after the 15-min incubation in cells pre-treated with either an inhibitory anti-CD91 mAb (second panel from the bottom) or unlabeled α2M (last panel) indicates that the uptake of α2M by HSG and SGEC is a specific process mediated by CD91 receptor. The right part of each figure represents the differential interference contrast (DIC) images of the same area (Original magnification × 400).

it as a central regulator of innate responses. Furthermore, the ability of CD91 to mediate the cross-presentation of the peptides carried by CD91 ligands to MHC molecules and thus, to elicit specific immune responses against them, suggests that this pathway might participate in the presentation of autoantigens released during apoptotic procedures to the immune system. Under this perspective, epithelial CD91 pathway might be implicated in the regulation of local immune responses. Elevated cell death occurs in the salivary glands of SS patients, resulting in the release of apoptotic material including autoantigens [27–30]. Thus, despite the fact that similar ductal CD91 expression was detected in SS patients and controls, in conditions of chronic inflammation, such as SS, the epithelial CD91 might mediate the endocytosis and presentation of completely distinct materials than in controls. Hence, this pathway might be implicated in the differential activation of SGEC in SS patients.

In this context, the interaction of CD91 receptor with calreticulin is of note. CD91 receptor/calreticulin pathway has been considered to play a key role in the removal of apoptotic cells [8,11–13]. Calreticulin is a molecular chaperone with diverse functions. It has the capacity to form complexes with antigenic peptides, which in turn are loaded to MHC molecules by CD91 receptor and become immunogenic. The co-localization of calreticulin and Ro/SSA autoantigen in apoptotic blebs suggests that calreticulin possibly participates in the Ro/La ribonucleoprotein complex [31,32], which is the major target of autoimmune responses in SS and systemic lupus erythematosus. Furthermore, calreticulin specifically binds to the major linear epitopes of the Ro60 autoantigen and results in enhanced Ro60-peptide antigenicity [33–35]. Thus, it would be tempting to hypothesize that the CD91 expressed by SGEC in SS participates in the antigen-presentation of Ro-autoantigens via interaction with the calreticulin pathway.

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