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RESEARCH ARTICLE



Potential of serum soluble CD93 as a biomarker for asthma in an ovalbumin-induced asthma murine model

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ABSTRACT

Background: CD93 is a membrane-associated glycoprotein, which can be released in a soluble form (sCD93) into the serum. CD93 has received renewed attention as a candidate biomarker of inflammation in various inflammatory and immune-mediated diseases, including asthma.

Objective: We aimed to evaluate the effects of airway inflammation on CD93 levels in murine models.

Methods: We established an ovalbumin (OVA)-induced acute asthma murine model (OVA model) and a lipopolysaccharide (LPS)-induced airway inflammation murine model (LPS model). Dexamethasone was administered by gavage to attenuate the airway inflammation.

Results: The OVA model demonstrated typical allergic asthma features with increased airway hyper-responsiveness, inflammatory cell infiltration, increased Th2 cytokine levels, compared to the control group. CD93 levels were decreased in lung homogenates and, respiratory epithelial cells, whereas serum sCD93 levels were increased in the OVA model, as compared to the control group. Dexamethasone reversed these effects of OVA. In contrast, in the LPS model, CD93 levels were not affected in neither respiratory epithelial cells nor serum.

Conclusions: Our findings demonstrate the potential of using sCD93 as a biomarker for allergic asthma.

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Airway; asthma; biomarker; CD93; inflammation; ovalbumin

Introduction

CD93 is a membrane-associated glycoprotein expressed on the surface of cells involved in the inflammatory cascade, which can also be released into the serum in a soluble form (sCD93) (Greenlee *et al.* 2008, Greenlee-Wacker *et al.* 2012). Although the function of CD93 is still not precisely elucidated, recent studies have revealed that CD93 regulates several processes associated with immunity and inflammation. Moreover, some studies have highlighted the potential roles of CD93 in human immune or inflammatory related diseases, including arthritis (Jeon *et al.* 2010), systemic lupus erythematosus (Moosig *et al.* 2006), and asthma (Sigari *et al.* 2016, Park *et al.* 2017). However, the molecular function of CD93 and the mechanisms by which CD93 plays a role in immunity and inflammation are not yet fully elucidated, and *in vitro* and *in vivo* studies in this regard are lacking.

Asthma is a chronic inflammatory airway disease featuring a T helper 2 (Th2)-dominant allergic inflammation response. Although some biomarkers for asthma have been investigated, including pulmonary function test parameters and sputum eosinophil and neutrophil counts (Bandyopadhyay *et al.* 2013, Moore *et al.* 2014), some asthma patients cannot undergo these tests because of severe dyspnoea, the absence of sputum or non-cooperation (Kim *et al.* 2017).

Therefore, many researchers have focused on identifying serological biomarkers for asthma (Ko *et al.* 2016, Agache and Rogozia 2017, Byeon *et al.* 2017). The peripheral blood eosinophil count has been traditionally used as a biomarker to reflect the severity of asthma (i.e. symptoms, hyper-responsiveness and lung function); however, unlike the sputum eosinophil count, the peripheral blood eosinophil count does not correlate with airway inflammation (Bousquet *et al.* 1990, Hastie *et al.* 2013). Serum periostin has also been proposed as a useful biomarker for severe asthma with Th2-dominant features (Woodruff *et al.* 2009), and human chitinase-3-like protein 1 (YKL-40) shows potential as a biomarker for airway inflammation (Ober *et al.* 2008). Nevertheless, these serological biomarkers have not been widely applied to date owing to limitations of their utility.

A few recent studies have highlighted sCD93 as a new potential biomarker, which can be easily measured in serum (Sigari *et al.* 2016, Park *et al.* 2017). These studies demonstrated that the serum levels of sCD93 are elevated during asthma exacerbation; but decrease after treatment of an asthma attack. In addition, Bohlson *et al.* demonstrated that lipopolysaccharide (LPS) stimulation induces the shedding of CD93 *in vitro* (Bohlson *et al.* 2005). However, the precise location of CD93 in the airway system, and the changes of CD93

expression in the lung tissue and serum after exposure to allergic or acute inflammatory stimuli remain unknown.

Therefore, the present study aimed to evaluate the location of CD93 expression in the lung tissue and to ascertain the effects of allergic or acute airway inflammation on CD93 levels, with and without treatment, using murine models induced with LPS or ovalbumin (OVA), respectively.

Clinical significance

- CD93 is expressed on the surface of respiratory epithelial cells in murine model.
- CD93 can be released to the serum after allergen exposure in a murine model
- CD93 shows potential as a biomarker in allergic asthma.

Materials and methods

Animal model designs

Female BALB/c mice, between 5- and 6-week-old (Orient, Daejeon, Korea), were maintained at conventional animal facilities under pathogen-free conditions. To establish the OVA-induced asthma murine model (OVA model), the mice were sensitized with 20 μ g OVA (Sigma–Aldrich, St Louis, MO) suspended in 1% aluminium hydroxide (Resorptar, Indergen, New York, NY) by intraperitoneal injection on days 1 and 14. The control group was treated in the same way, but with 20 μ g of saline. On days 21, 22 and 23, the OVA-sensitized mice were challenged intranasally with 30 μ L of OVA (1 mg/mL) in saline solution. Dexamethasone (9 α -fluoro-16 α -methylprednisolone; Sigma–Aldrich, St. Louis, MO; 3 mg/kg) or vehicle was administered by gavage on days 21, 23 and 25 to reverse the effects of OVA treatment.

To generate the LPS-induced airway inflammation murine model (LPS model), LPS (Sigma–Aldrich, St. Louis, MO; 1 mg/kg) was administered to the BALB/c mice intranasally on day 23. Dexamethasone was also used to treat the airway inflammation induced by LPS, as described above.

All mice were sacrificed after the last treatment administration on day 25 (Figure 1). All experimental protocols were

approved by the Animal Research Ethics Board of Yonsei University (Seoul, Korea).

Measurement of airway hyper-responsiveness

Airway hyper-responsiveness (AHR) to inhaled aerosolized methacholine (MCh; Sigma–Aldrich, St Louis, MO) was measured using a forced oscillation technique (FlexiVent; SCIREQ, Montreal, QC, Canada) on the day of sacrifice, as described previously (Lee *et al.* 2013). Aerosolized phosphate-buffered saline (PBS) or methacholine at varying concentrations (3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25.0 mg/mL and 50.0 mg/mL), was administered to the mice for 10 s via a nebulizer connected to a ventilator. AHR was then assessed based on measurements of airway resistance.

Inflammatory cell counting in bronchoalveolar lavage fluid

To collect bronchoalveolar lavage fluid (BALF), lung lavage was performed, using 1 mL of Hank's balanced solution (HBSS), through a tracheal tube. The recovered BALF was centrifuged and resuspended in 300 μ L HBSS. Total cell numbers were determined using a haemocytometer and trypan blue staining. BALF cells were centrifuged by cytocentrifugation (Cytospin 3; ThermoFisher Scientific, Waltham, MA) and were pelleted to cytospin slides. The slides were stained with haematoxylin and eosin (H&E Hemacolor®, Merck, Darmstadt, Germany) and a differential count of inflammatory cells was performed (200 cells per slide).

Histological analysis

The lung that was not used for BALF collection was fixed in 4% formalin and embedded in paraffin. Lung sections were cut into 3- to 4- μ m-thick slices and stained with H&E, periodic acid–Schiff (PAS), and Masson trichrome (M&T) for histological analysis. The slides were observed under a light microscope ($\times 40$ magnification).

Lung homogenate

After collecting the BALF, the remnant lung was resected and homogenized using a tissue homogenizer (Biospec Products, Bartlesville, OK) in lysis buffer and protease inhibitor solution (Sigma-Aldrich, St Louis, MO). After incubation and centrifugation, the supernatants were harvested and passed through a 0.45- μ m filter (Gelman Science, Ann Arbor, MI). The final preparation was stored at -20°C for cytokine measurements in line with a previous study (Lee *et al.* 2013).

Measurement of cytokine and CD93 levels

Concentrations of interleukin (IL)-13, IL-4, transforming growth factor (TGF)- β , interferon (IFN)- γ , and CD93 in the lung homogenates and serum were assessed by enzyme-linked immunosorbent assays (IL-3, IL-4 and TGF- β by R&D Systems, San Diego, CA; IFN- γ and CD93 by

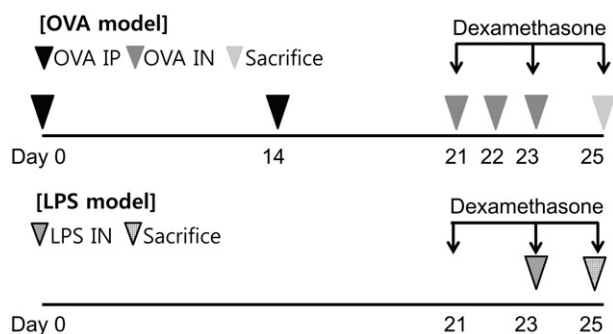


Figure 1. Scheme of animal model generation. The OVA model was established by two times of intraperitoneal injections of OVA and three intranasal OVA challenges. The LPS model was induced by intranasal LPS injection. Dexamethasone was administered by gavage for 3 days. All mice were sacrificed on the day of the last dexamethasone treatment. OVA: ovalbumin; IP: intraperitoneal injection; IN: intranasal inoculation; LPS: lipopolysaccharide.

Affymetrix eBioscience, Vienna, Austria) according to the manufacturer instructions. The detection range for CD93 was 3.3–400 ng/mL. All samples were assessed in duplicate.

Immunohistochemical staining of CD93

For immunohistochemistry, the slides were deparaffinized using xylene and washed. Hydrogen peroxide (3% in methanol) was used to inactivate endogenous peroxidases. The slides were then incubated with anti-CD93 antibody (ab198854, Abcam, Cambridge, MA) for 1 h, followed by 4°C for 24 h. After washing in PBS, the slides were treated with goat anti-rabbit IgG H&L (ab150077, Abcam) and incubated for 20 min at room temperature. Diaminobenzidine staining was then developed for 5 min. The slides were counterstained with haematoxylin and mounted using mounting medium.

Statistical analysis

All results are expressed as the mean $\pm$ standard error. We used parametric statistical analyses, because the total number of mice used in this study was 30 and the Kolmogorov–Smirnov and Shapiro–Wilk tests showed that almost all of parameters assessed followed a normal distribution. The AHR data were analysed using repeated-measures analysis of variance

(ANOVA), followed by a *post hoc* Bonferroni test. One-way ANOVA was performed to assess the significance of differences in the BALF cell count, cytokine levels and CD93 levels among groups. All statistical analyses were performed with IBM SPSS version 18.0 (SPSS Inc., Chicago, IL). *p* values <0.05 were considered statistically significant.

Results

OVA and LPS model establishment with and without dexamethasone treatment

Both the OVA and LPS models developed significant AHR, whereas the control group showed normal airway reactivity. Dexamethasone significantly ameliorated the AHR caused by OVA and LPS (Figure 2(A)).

BALF analysis revealed that the OVA model exhibited significant total cell and eosinophil proliferation. In contrast, the LPS model exhibited neutrophil-dominant proliferation. Dexamethasone ameliorated the BALF cell proliferation caused by OVA and LPS (Figure 2(B)).

Histological findings showed abundant infiltration of inflammatory cells and extensive proliferation of goblet cells in the OVA model, where only moderate proliferation of inflammatory cells was observed in the LPS model, as compared to

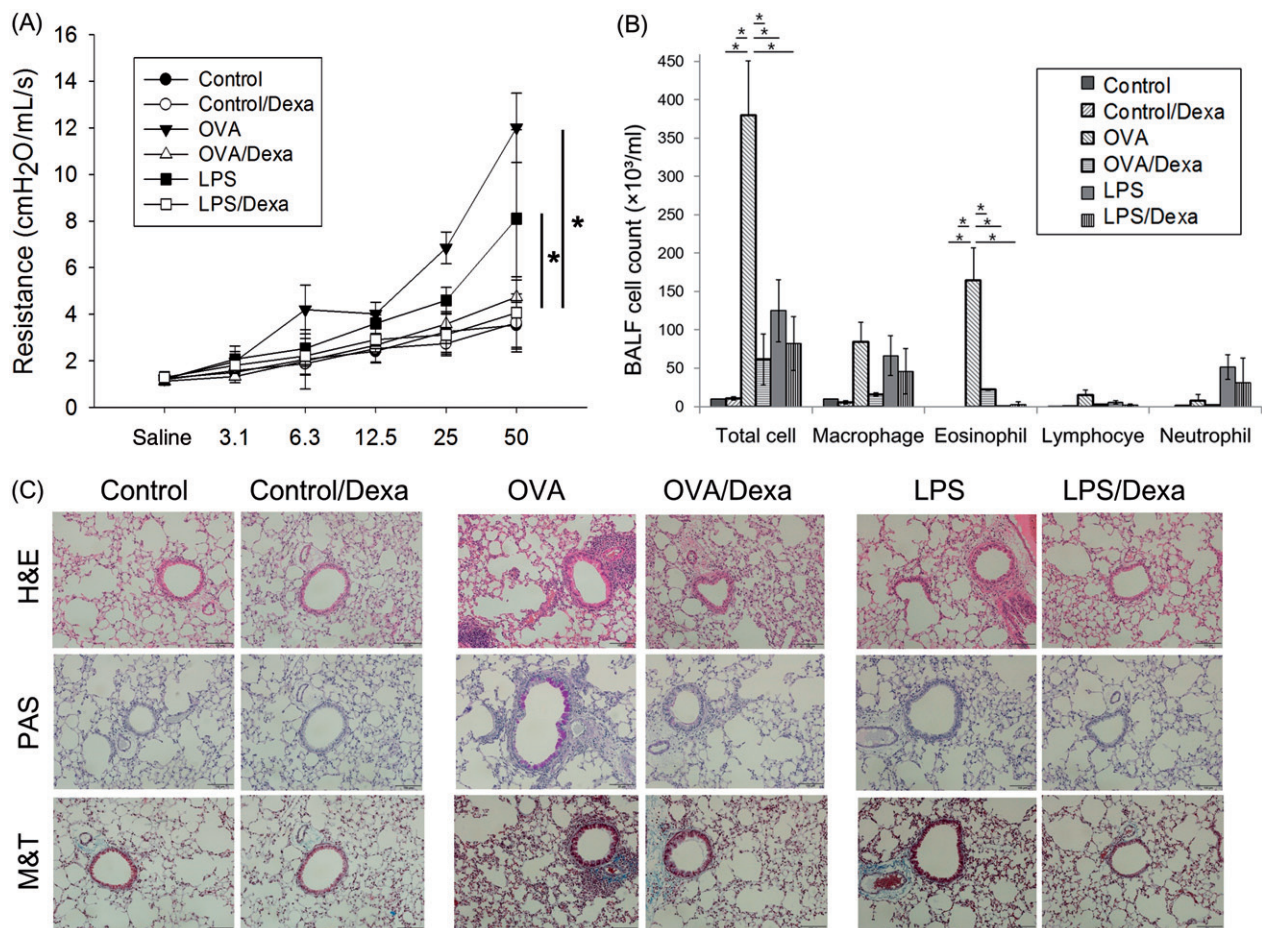


Figure 2. Airway hyperresponsiveness (AHR) determined by methacholine challenge showed that the OVA and LPS models developed significant AHR (A). BALF cell counts revealed that the OVA model exhibited eosinophil dominant inflammation, and the LPS model exhibited neutrophil dominant inflammation (B). Histologic analysis (H&E, PAS, and M&T; all magnification $\times 40$) showed inflammatory cell infiltration in the OVA and LPS models (C). In addition, dexamethasone administration ameliorated all of the above parameters. OVA: ovalbumin; LPS: lipopolysaccharide; Dexa: dexamethasone; H&E: haematoxylin and eosin; PAS: periodic acid–Schiff; M&T: Masson trichrome. **p* < 0.05.

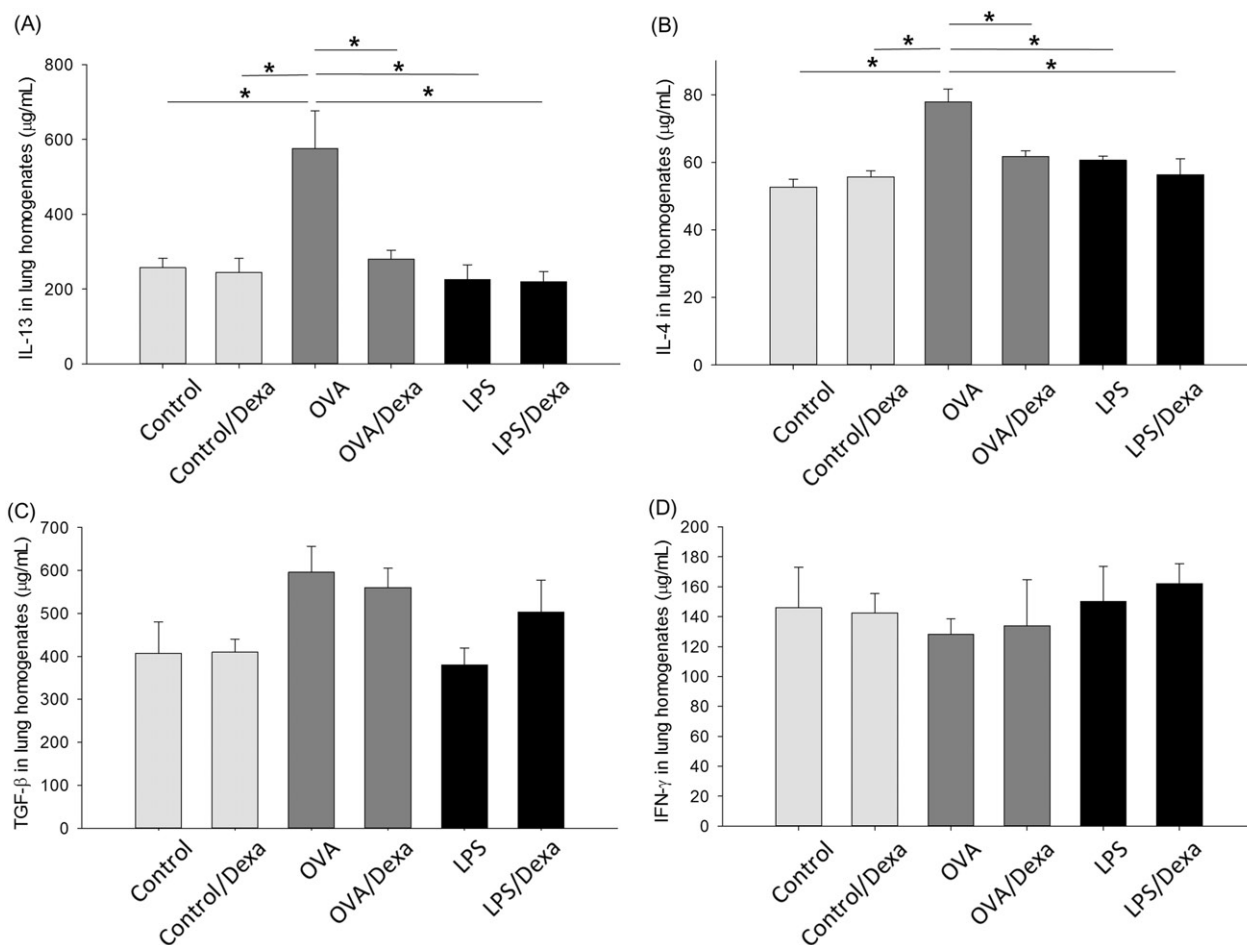


Figure 3. IL-13 (A), IL-4 (B), TGF- β (C), and IFN- γ (D) levels in lung homogenates showed that the OVA model and LPS model exhibited Th2-dominant and non-Th2-dominant features, respectively. In addition, dexamethasone administration decreased the levels of all the tested cytokine level. * $p < 0.05$.

the control group. Dexamethasone diminished the cell proliferation in both the OVA and LPS models (Figure 2(C)).

The OVA model showed Th2-dominant features, with increased levels of IL-13 and IL-4 compared to the control group. Dexamethasone significantly reversed these increases. By contrast, the LPS model showed non-Th2-dominant features (Figure 3).

Changes in CD93 levels in lung homogenates

The OVA model showed a significant decrease in CD93 levels in lung homogenates, as compared to the control group. Dexamethasone reversed these effects of OVA on lung tissues. In contrast, the LPS model did not show a significant change in CD93 levels in lung homogenates (Figure 4).

Changes in CD93 expression in lung tissues assessed by histological analysis

Immunohistochemical staining showed that CD93 expression in respiratory epithelial cells was decreased in the OVA model, as compared to that of the control group. Dexamethasone reversed these effects of OVA in respiratory epithelial cells. In contrast, the LPS model did not show any changes in CD93 expression in lung tissues (Figure 5).

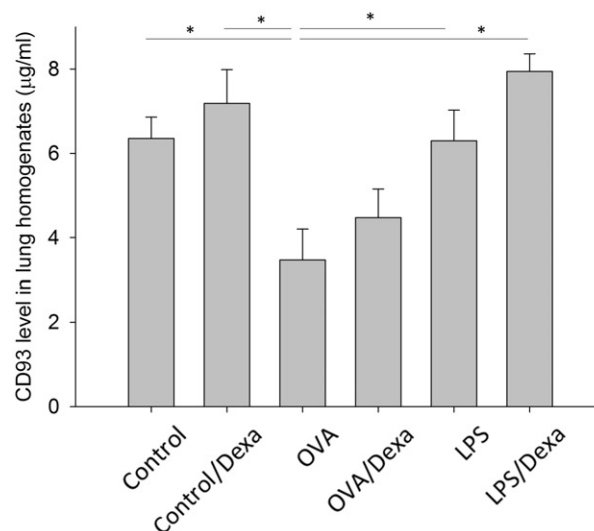


Figure 4. CD93 levels in lung homogenates were significantly elevated in the OVA model; but it decreased after dexamethasone treatment. OVA: ovalbumin; LPS: lipopolysaccharide; Dexa: dexamethasone. * $p < 0.05$.

Changes in serum sCD93 levels

The sCD93 level in the serum was increased in the OVA model, as compared to that of the control group, and dexamethasone reduced the sCD93 levels in the serum of

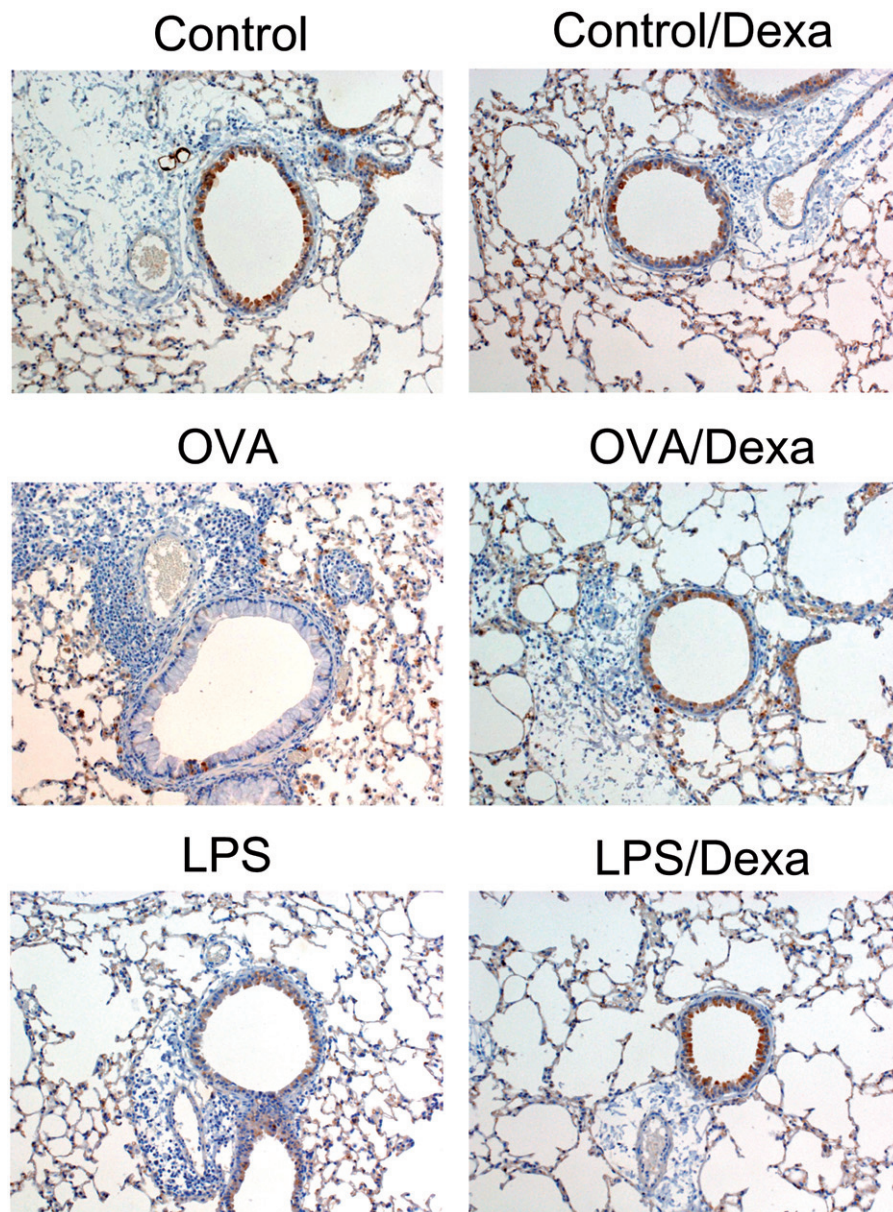


Figure 5. CD93 expression in respiratory epithelial cells assessed by histologic analysis (immunohistochemical staining with anti-CD93 antibody; all magnifications, $\times 40$). The CD93 expression was increased in the OVA mode, but decreased after dexamethasone treatment. OVA: ovalbumin; LPS: lipopolysaccharide; Dexa: dexamethasone.

these mice. However, LPS did not affect the levels of sCD93 in the serum (Figure 6).

Discussion

Here, we evaluated the effects of airway inflammation on CD93 expression in murine models, which have not been reported previously. We showed that the levels of CD93 in the respiratory epithelial cells and in serum change in opposite directions in an OVA-induced asthma murine model. This implies that CD93 in respiratory epithelial cells may be released into the serum after exposure to allergic inflammatory stimuli.

Bohlson *et al.* first reported that CD93 can be shed from the cell surface into the serum in response to various inflammatory stimuli, including dibutyrate, LPS and TNF- α (Bohlson *et al.* 2005). This shedding of CD93 is mediated by

metalloproteinase; and the cleavage sites are known to exist between P1 and P1' (Turk *et al.* 2001, Bohlson *et al.* 2005). However, allergic inflammatory stimuli such as OVA have never been suggested to induce the shedding of CD93. Our findings thus demonstrated that shedding of CD93 may be a biomarker for allergic inflammation. Recently, two studies in humans showed that levels of serum sCD93 are elevated during asthma exacerbation, supporting the main results of our study (Sigari *et al.* 2016, Park *et al.* 2017). However, the origin of the shed CD93 remains unclear, i.e., epithelial cells, endothelial cells, or both, because lung homogenates contain various cell types including both endothelial and epithelial cells. In addition, the mechanisms by which CD93 is shed to enter the systemic circulation is unknown. The further studies are needed to reveal these mechanism, and to determine whether CD93 is shed to the sputum or BALF.

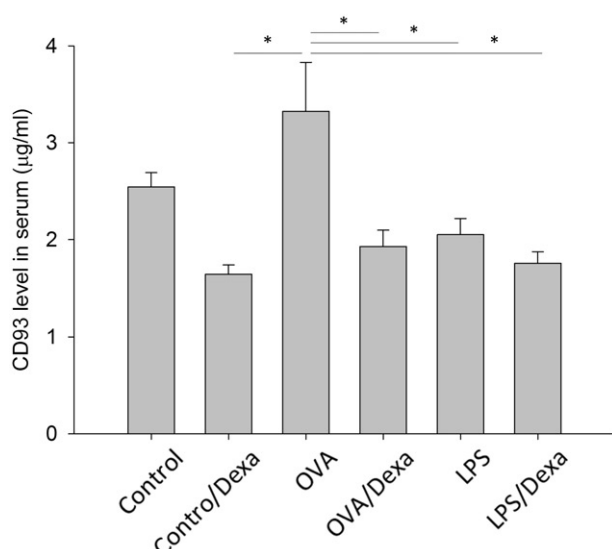


Figure 6. The serum CD93 level was increased in the OVA model; but was reduced after dexamethasone administration. OVA, ovalbumin; LPS, lipopolysaccharide; Dexa, dexamethasone. * $p < 0.05$.

Although originally described as a marker for developing B cells (McKearn *et al.* 1984, Allman *et al.* 2001), CD93 is now recognized as an inflammatory regulator expressed on various haematopoietic and inflammatory cells, including myeloid cells, lymphocytes, stem cells, and endothelial cells (Guan *et al.* 1994, Pilling *et al.* 2009, Greenlee-Wacker *et al.* 2012). The role of CD93 in the airway system has only recently been revealed. However, the location of expression of CD93 in the airway system has thus far remained unknown. In the present study, we elucidated the location of CD93 expression in the airway system and showed that CD93 is expressed on the surface of respiratory epithelial cells.

Dexamethasone reversed the effects of CD93 on respiratory epithelial cells and in the serum. Dexamethasone decreased the levels of CD93 in respiratory cells in the OVA model; however, it did not significantly decrease the levels of CD93 in control and LPS model mice. Therefore, we can assume that dexamethasone neither directly downregulated the CD93 level in respiratory cells, nor upregulated the CD93 levels in the serum. Overall, these findings indicate that dexamethasone simply reversed the allergic inflammation, which led to restoration of the decreased CD93 levels induced by OVA.

Although a previous *in vitro* study suggested that LPS stimulated the cleavage and shedding of CD93 (Bohlson *et al.* 2005), we found no significant effects of LPS on CD93 expression. Although we cannot fully explain this finding at present, we assume that it may be due to differences between the nature of *in vivo* and *in vitro* studies. We also consider that the interval between the LPS administration day and sacrifice day might have been too short to induce changes in CD93 levels in this study. Further *in vitro* and *in vivo* studies related to this issue are needed to reveal the effects of LPS on CD93.

Asthma is a chronic inflammatory airway disease for which there are currently few known serum biomarkers (Izuhara *et al.* 2016, Kim *et al.* 2016a, Kim *et al.* 2016b). CD93, a serum

biomarker, will be more helpful for asthma patients who are not able to undergo pulmonary function tests and sputum tests because of dyspnoea, the absence of sputum or old age. Although this study demonstrates the potential of serum sCD93 as a new biomarker for asthma, further *in vitro* and *in vivo* studies are needed to reveal the specific function and utility of serum sCD93 from a mechanistic point of view. In addition, clinical studies should be performed to define the utility of serum sCD93 as a biomarker for asthma.

There are some limitations of this study that should be mentioned. Firstly, we only observed the phenomenon of changes of CD93 but did not reveal the causal relationship. The specific mechanisms of shedding, such as the cleavage site, type of protease and associated mediators should be further evaluated. Secondly, we cannot confirm that CD93 levels in lung homogenates directly reflect the CD93 expression on respiratory epithelial cells. Lung homogenates contain small amounts of serum, inflammatory cells, hematopoietic cells, and endothelial cells. Therefore, the CD93 levels in lung homogenates may reflect the CD93 levels on respiratory epithelial cells as well as those on other cells. Lastly, it remains unknown whether human respiratory epithelial cells also express CD93.

Conclusions

We found that allergic inflammation decreased the levels of CD93 in respiratory epithelial cells, and increased the serum levels of CD93 using a mouse model. These effects were reversed by treatment with dexamethasone. In addition, LPS does not appear to affect the levels of CD93. Collectively, our findings demonstrate the potential of using sCD93 as a biomarker for allergic asthma.

Disclosure statement

The authors report no conflicts of interest

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