

Soluble CD200 correlates with interleukin-6 levels in sera of COPD patients – potential implication of the CD200/CD200R axis in the disease course

Running title: CD200 and COPD-like lung inflammation

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ABSTRACT

BACKGROUND: COPD represents a multifactorial lung disorder with high morbidity and mortality. Despite intensive research concerning the underlying disease mechanisms, the involvement of the CD200/CD200R axis in supporting or preventing the onset of COPD has not yet been addressed. Since the CD200/CD200R axis is crucially implicated in the maintenance of pulmonary immune homeostasis, we hypothesized that it might be involved in controlling the onset of COPD.

METHODS: To address this, we analyzed serum samples from COPD patients and normal controls for soluble (s) CD200 and correlated the data to COPD-relevant clinical parameters. In addition, basic studies were conducted in CD200-deficient and wild-type mice in which COPD-like inflammation was induced with elastase/LPS followed by lung and serum component analysis.

RESULTS: We observed a positive correlation between serum sCD200 and IL-6 levels as well as a trend towards a negative correlation of sCD200 with Vitamin D3 in COPD patients. Further investigations in mice revealed that despite elevated serum concentration of MMP-9 in CD200KO mice the early onset of COPD-like lung inflammation was similar in CD200-deficient and wild-type animals in terms of immune cell infiltration, emphysematous changes and mucus overproduction.

CONCLUSIONS: While our murine studies suggest that the co-inhibitory molecule CD200 does not appear to play a prominent role in the early onset of COPD-like features, correlation of sCD200 serum levels with COPD-related parameters in humans with established disease revealed that the CD200/CD200R axis may be mechanistically linked to the disease course in COPD patients.

Key words: CD200/CD200R axis, COPD, LPS/elastase model, CD200 deficient mice, MMP-9.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a complex lung ailment that is characterized by chronic airflow obstruction [1], small airway and vascular remodeling [2] and mucus overproduction [3]. The World Health Organization estimates COPD to be the 3rd leading cause of death by 2030 [4]. Pulmonary emphysema and chronic inflammation represents the clinical hallmarks of COPD, marked by alveolar space enlargement [5] and lung parenchymatic destruction [6]. This persistent pulmonary inflammation comprises mainly of neutrophils, macrophages [8] and lymphocytes [10]. These cellular components secrete an array of inflammatory cytokines and matrix-metalloproteinases (MMPs) to potentiate emphysema formation and systemic spillage is usually evident during disease progression [7]. Together, pulmonary tissue damage mediated by mucosal immune cells following an initial inflammatory trigger plays a principal role in the disease progression in COPD patients [8]. Thus, improved understanding concerning immunological mechanisms are needed for the development of targeted immune-intervention.

The CD200/CD200 receptor (CD200R) axis has been reported to control myeloid cell activation [9, 10] and to be crucially involved in the maintenance of lung immune homeostasis [11] in both human and mice [12, 13]. The CD200 molecule is expressed on airways epithelial cells, endothelial cells and T cells [9, 14], whereas the CD200R molecule is predominantly expressed on airway macrophages and neutrophils [13].

In human studies, a new COPD therapeutic, adenosine A_{2B} agonist, enhances CD200 expression in human airway epithelial cells, indicating the anti-inflammatory quality of the drug [15]. There exist soluble (s) forms of CD200 and its receptor CD200R in human sera [16] that are produced either by membrane shedding [17] or by mRNA splicing [18]. Several clinical investigations have demonstrated elevated serum levels of sCD200 in patients with type 2

diabetic foot and nephropathy [19], bullous pemphigoid [20], psoriasis vulgaris [21], SLE [16] and Samter's syndrome [22]. The fact that the level of sCD200 in serum is elevated in several diseases might indicate both a possible implication of the CD200/CD200R axis in inflammatory diseases and also imply a potential use of sCD200 as a biomarker. In animal studies, CD200^{-/-} mice were shown to develop excessive lung inflammation with enhanced neutrophils [23] and T lymphocytes [24] infiltration into the lung, and thereby succumb to severe tissue damage following pulmonary infection [13].

In this study, we analyzed serum concentrations of sCD200 in COPD patients and normal controls and correlated the data with COPD-relevant clinical parameters. Next to analyses in patients with advanced-stage COPD, we experimentally addressed whether the CD200/CD200R regulatory axis might be functionally involved in immune cell control in the lung during the developmental phase of COPD.

MATERIALS & METHODS

Human subjects

50 serum samples were collected from COPD patients (36 men, 14 women) and 29 serum samples were collected from age- and gender matched normal controls (19 men, 10 women) at the Pneumology Unit, Magdeburg University Hospital, Magdeburg, Germany and the Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany. The average age of the patients and normal controls were 66.7 and 61.6 years, respectively. All subjects were given the written informed consent and the study protocol was performed in accordance with the declaration of Helsinki and approved by the local ethic committee of the University of Magdeburg, Germany (Aktenzeichen 12/11). The characteristics of COPD patients and normal donors are given (Table 1).

Mice

7-10 weeks old female CD200KO C57BL/6 mice (Ophthalmology Research Unit, University of Bristol, United Kingdom) and age- and sex-matched C57BL/6 wild-type mice (Harlan Winkelmann, Rossdorf, Germany) were used. CD200KO (n=14) and wild-type mice (n=28) were maintained under specific pathogen-free conditions in the animal facility at HZI, Braunschweig, Germany. Animal experiments were conducted according to the institutional guidelines and the study was ethically approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (33.19-42502-04-15/1750).

Elastase/LPS exposure

COPD-like features were induced in both CD200KO and C57BL/6 wild-type mice by repeated intranasal exposure to elastase and lipopolysaccharide (LPS) as described previously [25], for two and four consecutive weeks (Fig. 1A). As negative control, mice were exposed to 50%

glycerol (carrier, elastase preparation) and phosphate-buffered saline (PBS) (LPS solvent). Mice were sacrificed one week after the final exposure to LPS or PBS for further investigations.

Lung Volumetry and Histology

Excised lungs were immersion fixed in 4 % formaldehyde for up to 48 hrs. The volume of each lung was estimated by Scherle's displacement method [26] and the lung volume was normalized to the respective total body weight of the mice. For histology, paraffin-embedded tissues were cut at 3 μ m and tissue sections were mounted on SuperfrostPlus adhesive glass slides (Menzel-Gläser; Braunschweig, Germany). Tissue sections were stained with hematoxylin and eosin (H&E) for histological examination. Periodic acid-Schiff (PAS) reaction was performed to stain mucins. To evaluate histopathological lesion, the inflammatory pattern (peribronchial, perivascular) and the immune cell compositions were determined and the occurrence of neutrophils, alveolar macrophages and lymphocytes was scored (none=0, minimal=1, mild=2, moderate=3, severe=4). A semi-quantitative evaluation of the mucus production was performed, the cellular staining intensity (weak=1/strong=2) and the positive cells within bronchi (none=0, scattered=1, multifocal=2, diffuse=3) were assessed. Finally, emphysematous changes were determined (none=0, minimal=1, mild=2, moderate=3, severe=4).

ELISA

Serum levels of total MMP-9 (1:50 dilutions) (Cat. # MMPT90) (R&D systems) in elastase/LPS exposed mice were determined according to the manufacturer's instructions. Serum levels of sCD200 (Cat. # SEK 10886) (Sino Biologicals Inc.) and TNF α (Cat. # DTA00C) (R&D systems) in COPD patients and normal controls were quantified according to the manufacturer's instructions. All analyses were done in duplicate.

Immunoturbidimetry

Serum levels of high-sensitivity C-reactive protein (hsCRP) in COPD patients were quantified by turbidimetry (800 nm/570 nm) using a cobas c 311 analyzer (Roche).

Assay for cytokine and vitamin profiles

Quantification of vitamin D ((Vitamin-D-1, 25-OH) and IL-6 cytokine in the serum samples of COPD patients was performed as routine procedure at the pneumology unit, University Hospital Magdeburg.

Statistical analysis

Non-parametric Mann-Whitney test was used to compare elastase/LPS exposed mice to glycerol/PBS exposed control animals, elastase/LPS exposed CD200KO to C57BL/6 wild-type controls, serum MMP-9 levels in the mouse model and the serum sCD200 levels in COPD patients compared to normal controls. Two-tailed p -value <0.05 were considered significant.

RESULTS

Serum sCD200 concentration is positively correlated to the abundance of the pro-inflammatory cytokine IL-6 in human COPD patients

Since increased serum levels of sCD200 in patients with inflammatory diseases have been shown [20, 21], we sought to analyze sera of COPD patients and normal controls for the presence of sCD200. While sCD200 was well detectable in sera of normal and diseased donors, we did not observe any significant differences (Fig 1). However, further correlation of COPD-relevant clinical parameters with serum sCD200 levels revealed a significant positive correlation between sCD200 and IL-6 (Table 2). A positive correlation was also observed with

the age of the patient, but not in normal controls (Table 2). Although not significant, there was a trend towards a negative correlation with vitamin-D-1,25-OH in COPD patients. No correlation was found between sCD200 and TNF α , CRP and GOLD stage of the patients.

Induction of a COPD-like phenotype in mice by repetitive elastase/LPS exposure

Since serum sCD200 were correlated with COPD-related parameters in patients with established COPD, we next aimed to study the role of the CD200/CD200R axis in early stages of COPD development. Here, we utilized a previously described elastase/LPS mouse model [25] that resulted in hallmark features of COPD such as immune cell infiltration, emphysematous changes and mucus overproduction. Accordingly, 4 weeks of elastase/LPS exposure (Fig 2A) induced marked inflammation in C57BL/6 mice with significant neutrophils (Fig 2B) and lymphocytes influx (Fig 2C), overall airway inflammation, increased alveolar macrophages influx and emphysematous changes (Fig 2D and E), although the latter two parameters did not reach statistical significance. However, we observed significantly increased mucin production (Fig 2D and 2E). Thus, elastase/LPS exposure resulted in the induction of COPD-like features in wild-type mice and could be used to study the role of the CD200/CD200R axis in COPD-like lung inflammation.

CD200-deficiency does not affect the onset of COPD-like features in mice following elastase/LPS exposure

Since the CD200/CD200R axis was reported to be involved in lung immune homeostasis, we expected an early and augmented inflammatory immune cell infiltration during the developmental stage of COPD-like inflammation in the absence of CD200. To experimentally address this hypothesis, we analyzed for COPD-related inflammatory alterations in the lung at 2 weeks and 4 weeks post initial elastase/LPS treatment. Unexpectedly, we observed a slight (but not significant) reduction in neutrophil (Fig 3A), lymphocytes (Fig 3B) and alveolar macrophage

(Fig 3E) recruitment and an overall slightly reduced inflammation score (Fig 3E) in CD200KO mice compared to control mice (Fig 3D) (for week 4). No differences were observed regarding emphysematous changes (Fig 3E), lung volume (Fig 3C) and mucus production (Fig 3E) between CD200-deficient and wild-type animals. Thus, the CD200/CD200R axis does not seem to play a dominant role in the early development of a COPD-like phenotype in mice.

Increased serum MMP-9 levels were detected in CD200-deficient mice following elastase/LPS exposure

Although we did not observe obvious morphological differences in CD200KO and wild-type animals following elastase/LPS exposure, we sought to determine the pro-inflammatory cytokine/chemokine (IL-1 β , IL-6, TNF- α , IL-10 as well as the chemoattractant MCP-1) response in the bronchoalveolar lavage fluid (BALF). However, only nominal concentrations of these cytokines were detectable and no differences between the CD200KO and wild-type mice were observed (data not shown). Since MMPs are crucially involved in the development of emphysematous lesion in COPD, we also analyzed the levels of MMP-9, one of the lead MMPs in emphysema formation [27]. Indeed, we observed significantly increased concentrations of MMP-9 in 4 weeks elastase/LPS exposed CD200KO mice compared to wild-type control animals (Fig 4). Thus, serum MMP-9 concentration in CD200KO mice is elevated compared to wild-type mice in animals with established COPD-like features.

DISCUSSION

To our knowledge, this is the first study to address the role of the immune-inhibitory CD200 molecule in COPD patients as well as in COPD-like lung inflammation in mice. Our rationale to conduct the study was based on the well-known critical role of the CD200/CD200R axis in controlling lung inflammation and maintenance of lung homeostasis [13] predominantly by inhibition of airway macrophage activation [11]. Since therapeutic intervention aiming at targeted inhibition of neutrophil and macrophage activation is given a priority in COPD treatment regimen, we sought to determine whether CD200 would be mechanistically linked to loss of pulmonary immune homeostasis and thereby may represent a novel candidate molecule for improved treatments.

To unravel the potential role of CD200 in human COPD, we first addressed whether serum levels of sCD200 would be altered in COPD patients compared to normal controls. Although sCD200 was shown to influence regulatory T cells (Tregs) expansion [28], the exact function of serum sCD200 in human diseases remains obscure. For instance, elevated serum sCD200 levels were correlated with disease severity in patients with inflammatory skin diseases [29], whereas it correlated with myeloid-derived suppressive cells in glioblastoma patients [30], pointing to both pro- and anti-inflammatory effects. Nevertheless, we could not detect any significant alterations in sCD200 levels in COPD patients. However, we may speculate that multifactorial stimuli like smoking or recurrent infections may result in decreased CD200 cell-surface expression, which may limit the membrane shedding and account for reduced sCD200 in COPD patients. Alternatively, the role of CD200 might be negligible in COPD. Intriguingly, a positive correlation of sCD200 with the pro-inflammatory cytokine IL-6 and age and a trend for inverse relation with vitamin D3 provided a first hint for a potential pro-inflammatory role of sCD200 in COPD pathogenesis. Vitamin D3 consumption was already shown to induce CD200

expression in airway immune cells, which was impaired in patients with chronic lung disease, suggesting the role of vitamin D to restrain airway inflammation [20]. One possible explanation could be that circulating sCD200 might block the cell-surface interactions between CD200 and CD200R to mediate their immune-inhibitory functions. Functional studies are required to prove this notion. Also, this study lacks the required power of analysis, which could be substantiated with a larger COPD cohort.

To further explore the role of the CD200/CD200R axis in COPD, we utilized the elastase/LPS exposure model to induce COPD-like lung inflammation in mice [25]. The obvious advantage of this model is that the COPD-like phenotype develops relatively fast and essentially mimics all hallmark features of human COPD. Accordingly, elastase/LPS treatment led to inflammation, especially mediated by neutrophils, macrophages and lymphocytes, development of emphysematous lesions and robust mucus over-production.

Since studies with CD200-deficient mice revealed a massive accumulation and activation of myeloid cells in various disease models [9, 31], we also expected CD200KO mice to exhibit increased myeloid cell influx following elastase/LPS exposure and a more severe COPD progression. Contradictorily, no significant differences in COPD-relevant histopathological parameters were observed. One possible reason could be the existence of other lung regulatory mechanisms that might compensate for the functional loss of the CD200 molecule and may control the myeloid cell activation. Indeed, such compensatory mechanisms underlining the phenomenon of biological robustness of immune-regulatory circuits have been reported [32-34]. Another possible explanation could be that in addition to CD200 other, yet unknown, inhibitory ligands for CD200R may exist. This is not irrelevant since only few years ago, a high-throughput screening of a receptor array identified ICOS-L as a ligand for CD28 on human T cells in addition to the well-established receptor molecule ICOS [35].

Concerning our observation that, in general, disease progression monitored in our mouse model might not entirely reflect the disease course in human COPD, we may speculate that this could be related to the fact that our laboratory mice are kept under specific pathogen-free conditions, i.e. in contrast to humans they exhibit a very limited microbial flora and are less exposed to internal antigenic triggers and infectious agents, which might act as external triggers, to stimulate disease progression, as seen in COPD patients.

Despite negligible histological effects, we detected increased serum levels of MMP-9 in CD200-deficient animals. The main sources of MMP-9 are usually neutrophils, alveolar macrophages [36] and lung epithelial cells [37]. A possible reason for increased serum MMP-9 levels could be that the absence of the CD200 results in enhanced susceptibility of lung epithelial cells to the action of elastase and LPS. In support of this, it has been shown that the lack of PD-L1, another co-inhibitory ligand binding to the PD-1 molecule on T cells, resulted in impaired inhibition of MMP-9 secretion in neutrophils [38]. Importantly, we have analyzed total secreted MMP-9 that includes both inactive and active forms of serum MMP-9. Here, the increased serum MMP-9 may account for the inactive form, which might not necessarily result in enhanced emphysematous changes.

In this study, we exclusively focused on the question whether CD200-deficiency would alter the development of COPD-like inflammation in mice. Another approach would be either to induce overexpression of CD200 or alternatively to modulate CD200R by fusion protein or antibodies to study the implication of CD200/CD200R axis on the disease course. The rationale behind targeted modulation of the CD200R is that the receptor has been shown to be upregulated on pulmonary myeloid cells even under strong LPS stimulus [13]. Furthermore, unlike other receptor-ligand pairs [39, 40], CD200-bearing cells do not mediate downstream signals due to its short cytoplasmic tail that has no signaling motifs. Hence the central response following

CD200/CD200R interaction is mediated through CD200R-bearing myeloid cells [9]. Therefore, we suggest that future investigations should consider fine tuning of CD200R expression on myeloid cells for achieving direct signaling impact in macrophages and neutrophils on disease outcomes. Together, our data obtained in the murine elastase/LPS COPD model implied that the CD200/CD200R axis does, if at all, only play a minor role in COPD development.

CONCLUSIONS

This study demonstrated a positive correlation between serum sCD200 and IL-6 and age of COPD patients. Despite strong evidences for the implication of the CD200/CD200R axis in preservation of lung homoeostasis, no obvious impact of CD200-deficiency on the development of murine COPD-like lung inflammation was observed. Nevertheless, we do not exclude that investigations performed in cigarette smoke-induced COPD models or utilizing antibody-mediated blockade of the CD200R could still uncover a potential mechanistic link of the CD200/CD200R axis to the multifactorial disease course in COPD patients.

ABBREVIATIONS

COPD- Chronic Obstructive Pulmonary Diseases; CRP- C-Reactive Protein; GOLD- Global Initiative for Chronic Obstructive Lung disease; H&E- hematoxylin and eosin; IL- Interleukin; KO- Knock Out; LPS- Lipopolysaccharides; MMP-9- Matrix Metalloproteinases 9; PAS- Periodic Acid-Schiff; s- soluble; SEM- Standard Error Mean

CONFLICT OF INTERESTS

None

AUTHOR´S CONTRIBUTION

Conception and design: PS, DB; Experimental work and data analysis: PS, AB, MG, DC, CS;

Data Interpretation and manuscript writing: PS, AB, AG, ADG, ADD, JS, DB. All author´s read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Comparable sCD200 serum levels in COPD patients and normal controls.

Graphs represent circulating serum levels of sCD200 from COPD patients (n=50) and normal controls (n=29). The dots represent data obtained for individual patients and control subjects. *P*-values were calculated using the non-parametric Mann-Whitney test.

Figure 2. Induction of a COPD-like phenotype in C57BL/6 wild type mice by repeated elastase and LPS exposure

(A) Schematic representation of the COPD model based on repetitive intranasal administration of elastase/ LPS for 4 weeks. (B) Histological neutrophil and (C) lymphocyte scoring in the lung tissues of elastase/LPS exposed mice (n=10) and control treated mice (n=8). (D) Representative histopathology of COPD-like phenotype (a-h) in wild type mice, 5 weeks after initial treatment: (a-d) elastase and LPS treated mice, (a) inflammation including neutrophils and lymphocytes: score: 3 (moderate), H&E staining, scale bar: 50µm, inlay bar: 20µm; (b) alveolar macrophages infiltration: score 2,5 (mild to moderate), H&E staining, scale bar: 50µm; (c) emphysema: score 3 (moderate), H&E staining, scale bar: 100µm; (d) mucin staining in large bronchi: score 2 (strong cellular signal) and 2,5 (multifocal to diffuse), PAS-reaction, scale bar: 100µm, inlay bar: 20µm. (e-h) Glycerol and PBS treated mice, (e) inflammation: score 0 (none), H&E staining, scale bar: 50µm; (f) alveolar macrophages infiltration: score 0 (none), H&E staining, scale bar: 50µm; (g) emphysema: score 1 (minimal), H&E staining, scale bar: 100µm; (h) mucin staining in large bronchi: score 1 (weak) and 2 (multifocal), PAS-reaction, scale bar: 100µm. (E) Represents histological inflammation (neutrophils, lymphocytes, alveolar macrophages), alveolar macrophages, emphysema and mucus production scoring in the lung tissues between COPD induced (n=10) and control mice (n=8). The dots represent the data obtained for individual mice. Data obtained were pooled from

two independent experiments. *P*-values were calculated using non-parametric Mann-Whitney test.

Figure 3. *CD200-deficiency does not affect the onset of COPD-like lung inflammation in elastase/LPS-exposed mice.* (A) Histological neutrophil and (B) lymphocyte scoring in the lung tissues of elastase/LPS exposed CD200KO (n=7) and wild-type mice (n=5) following 2 and 4 weeks of treatment. (C) Lung volume (in %, Scherle method) in elastase/LPS exposed CD200KO mice (n=7) and wild-type (n=5) following 2 and 4 weeks of treatment. (D) Representative histopathology of COPD-like phenotype (a-h), 5 weeks after initial treatment with elastase and LPS in (a-d) wild type mice and in (e-h) CD200KO mice: (a) inflammation including neutrophils and lymphocytes: score 3 (moderate), H&E staining, scale bar: 50µm, inlay bar: 20µm; (b) alveolar macrophages infiltration: score 2,5 (mild to moderate), H&E staining, scale bar: 50µm; (c) emphysema: score 3 (moderate), H&E staining, scale bar: 100µm; (d) mucin staining in large bronchi: score 2 (strong) and 2,5 (multifocal to diffuse), PAS-reaction, scale bar: 100µm, inlay bar: 20µm. (e) inflammation: score 2 (mild), H&E staining, scale bar: 50µm, inlay bar: 20µm, (b) alveolar macrophages infiltration: score 2,5 (mild to moderate), H&E staining, scale bar: 50µm; (c) emphysema: score 3 (moderate), H&E staining, scale bar: 50µm, inlay bar: 20µm; (d) mucin staining: score 2 (strong) and 2 (multifocal), PAS-reaction, scale bar: 100µm, inlay bar: 20µm. The bar graphs represents (E) inflammation (neutrophils, lymphocytes, alveolar macrophages), alveolar macrophages, emphysema and mucus production scoring in the lung tissues between CD200KO COPD induced mice (n=7) and wild-type COPD induced (n=5) on week 2 and 4. The bar graphs are represented as a mean with SEM. *P*-values were calculated using non-parametric Mann-Whitney test.

Figure 4. *CD200-deficiency is associated with increased serum levels of MMP-9 in mice following 4 weeks of elastase/LPS exposure.* Graphs represent serum levels of total MMP-9

of elastase/LPS exposed CD200KO (n=7) and wild-type mice (n=5) after 2 (white bars) and 4 (black bars) weeks treatment. Bar graphs are represented as mean with SEM. *P*-values were calculated using the non-parametric Mann-Whitney test.