An evaluation of CD39 as a novel immunoregulatory mechanism invoked by COPD

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ABSTRACT

Acute exacerbations of chronic obstructive pulmonary disease (AECOPD) are characterized by increased pulmonary and systemic inflammation and commonly caused by bacterial and/or viral infection. Little is known about the T-cell dysregulation in AECOPD that promotes these outcomes. CD39 is an ectonucleotidase able to hydrolyse adenosine triphosphate to create adenosine that may inhibit T-cell responses in patients with AECOPD. Here T-cell expression of CD39 measured by flow cytometry was higher in AECOPD patients than stable COPD patients or healthy controls. Higher expression of CD39 was associated with higher levels of plasma soluble tumor necrosis factor receptor but lower interferon-γ (IFNγ) levels in supernatants from staphylococcal enterotoxin-B stimulated peripheral blood mononuclear cells. This links increased expression of CD39 with systemic inflammation and impaired T-cell responses (e.g. IFNγ). The blockade of CD39 pathways may be a novel approach to the control of AECOPD, reducing the dependency on antibiotics.

Keywords: AECOPD; CD39; COPD; IFNγ; T-cell

1. Introduction

Chronic obstructive pulmonary disease (COPD) is amongst the top five causes of global morbidity and mortality. COPD is characterized by chronic airway and systemic inflammation and so predisposes patients to ischemic heart disease, vascular disease, muscle wasting and cachexia [1, 2]. COPD patients may also experience acute exacerbations (AECOPD), commonly caused by infections [3]. AECOPD can accelerate the decline in lung function [4], but the associated immune dysregulation is not well understood. AECOPD may be an immune-deficient state reflecting an impaired T-helper type I (Th1) responses to infectious agents (e.g. decreased production of IFNγ) [5-9]. There are several anti-inflammatory molecules, including CD39 that may be highly expressed in AECOPD that may inhibit IFNγ responses.

CD39 is an ectonucleotidase which generates adenosine monophosphate (AMP) from adenosine triphosphate/adenosine diphosphate (ATP/ADP). CD73 (another ectonucleotidase) then converts AMP to adenosine, an anti-inflammatory molecule that can inhibit the function of $CD4^+$ and $CD8^+$ T-cells and natural killer cells [10]. CD39 is highly expressed by regulatory T-cells (Tregs) and is important for their immune-suppressive function [11, 12]. Inflammation usually results in the release of ATP that is converted to adenosine by Tregs via the CD39/CD73 pathway. The binding of adenosine to A_{2A} receptors downregulates the activity of effector T-cells but promotes the expansion of Tregs and their suppression of effector T-cell proliferation [13].

Therefore, CD39 and CD73 are important in the regulation of immune responses by inhibiting the ATP-driven pro-inflammatory immune cell activity and promote an anti-inflammatory state mediated by adenosine [10]. Since AECOPD may be an immunodeficient state, we hypothesized that chronic inflammation induces the expression of CD39 that then inhibits protective effector T-cell responses (e.g. IFNγ production) against bacteria in AECOPD.

2. Material and methods

2.1 Study subjects

AECOPD patients (n=21) admitted to the Royal Perth Hospital Emergency Department (Western Australia) were recruited. Stable COPD (sCOPD) patients who were previous smokers (>15 pack-years and ceased smoking >5 years earlier) from a dedicated Royal Perth Hospital COPD clinic (n=33) and healthy age-matched, non-smoking subjects with no clinical evidence of COPD were also included as controls (n=33). The diagnosis and severity of COPD was categorised by a respiratory physician according to the GOLD criteria (Stages 2-4). All patients had been treated with anticholinergic drugs, long-acting beta agonists and inhaled corticosteroids but none were receiving systemic corticosteroids or had diabetes, neuromuscular, allergic or rheumatological disease at the time of sampling. The study was approved by the Ethics Committee at the Royal Perth Hospital and all participants gave informed consent.

2.2 Sample and data collection

Peripheral blood mononuclear cells (PBMC) were isolated from blood collected in lithium heparin tubes by Ficoll gradient centrifugation and cryopreserved in 10% dimethyl sulfoxide/fetal calf serum. Plasma was stored at -80^{o}C. Plasma levels of IL-6, C-reactive protein (CRP) and sTNFR were measured by ELISA (R&D Systems, Minneapolis, MN). Proportions of $CD39^{+}$ T-cells were quantified by flow cytometry after staining PBMC with anti-CD3-APC-H7, CD4-V500, CD8-PerCP-Cy5.5, CD39-FITC, CD28-PE-Cy7 and PD-1-APC antibodies (BD Biosciences, San Jose, CA). Intracytoplasmic staining was performed using the BD Pharmingen^{TM} FoxP3 buffer set and FoxP3-PE antibody (BD Biosciences). 200,000 events were acquired using a BD FACSCanto II cytometer and analyses were done with FlowJo v5.7.2 software (Tree Star, Ashland, OR). Gating for T-cell subsets and expression of CD39 is shown in Figure 1. Concentrations of IFNγ, TNFα, IL-6, IL-10 (BD Biosciences) and IL-17 (eBioscience, San Diego, CA) were measured by ELISA in supernatants from PBMC ($2x10^6$ cells/mL) cultured with SEB (1μg/mL; Sigma-Aldrich, St. Louis, MO) for 24hr.

2.3 Data Analysis

Non-parametric Mann-Whitney tests were used to compare groups. Correlations were assessed using Spearman's rank correlation coefficient. Statistical analyses were performed with Graphpad Prism 5.04 software (La Jolla, CA). Statistically significant differences ($p<0.05$) are indicated in the figures and $p=0.05$-0.10 is noted as marking a trend.

3. Results

3.1 AECOPD patients exhibited systemic inflammation but decreased SEB-induced IFNγ production

Stable COPD (sCOPD) and AECOPD patients had elevated plasma levels of CRP ($p=0.004$ & $p<0.001$, respectively), IL-6 ($p=0.001$ & $p=0.003$, resp.) and sTNFR ($p=0.007$ & $p<0.001$, resp.) when compared to healthy controls (Figure 2, A-C). AECOPD patients had marginally higher plasma CRP ($p=0.07$), IL-6 ($p=0.09$) and sTNFR ($p=0.04$) than sCOPD patients.

Following stimulation with SEB, levels of IFNγ, TNFα and IL-6 was lower in PBMC from AECOPD patients than sCOPD patients ($p=0.0001$-0.002) and healthy controls ($p=0.0004$-0.005) (Figure 2, D-F). The production of IL-17 and IL-10 was lower in AECOPD patients than controls ($p=0.05$ for both) and sCOPD patients ($p=0.06$ and $p=0.002$, respectively) (Figure 2G and 2H). Level of plasma sTNFR showed weak to moderate negative associations with the levels of IFNγ ($r=-0.23$, $p=0.07$), IL-6 ($r=-0.23$, $p=0.07$), IL-17 ($r=-0.31$, $p=0.04$) and IL-10 ($r=-0.32$, $p=0.02$) in culture supernatants.

3.2 Expression of CD39 on T-cells correlates directly with plasma sTNFR and inversely with IFNγ production by T-cells

Proportions of circulating $CD39^+$ $CD4^+$ and $CD8^+$ T-cells, Tregs ($Foxp3^+$ $CD4^+$ T-cells) and non-Tregs ($Foxp3^-$ $CD4^+$ T-cells) were higher in AECOPD patients than sCOPD patients (Figure 3A). Compared to healthy controls, AECOPD patients exhibited increased proportion of $CD39^+$ $CD8^+$ T-

cells (p=0.008) and marginal higher proportion of $CD39^+$ $CD4^+$ T-cells (p=0.08). Furthermore, plasma sTNFR correlated directly with proportions of $CD39^+$ $CD4^+$ T-cells (r=0.25, p=0.048) and $CD8^+$ T-cells (r=0.28, p=0.027). The proportion of $CD39^+$ T-cells correlated inversely with the levels of IFNγ produced by SEB-stimulated PBMC (Figure 3, B-E), but not with levels of TNFα, IL-6, IL-17 and IL-10 (data not shown).

4. Discussion

This study describes for the first time the expression of CD39 on circulating T-cell subsets in relation to systemic inflammation and cytokine responses of patients with stable COPD or AECOPD. Our data demonstrated that the proportion of CD39-expressing $CD4^+$, $CD8^+$, $Foxp3^+$ (Tregs) and $FoxP3^-$ T-cells were increased in the circulation of AECOPD patients. This increase was associated with systemic inflammation as evidenced by a correlation with plasma levels of sTNFR. Furthermore, PBMC from AECOPD patients had impaired production of cytokines important for bacterial clearance such as IFNγ, TNFα, IL-6 and IL-17. Proportions of $CD39^+$ T-cells correlated inversely with SEB-induced IFNγ responses. This is in line with the findings with other inflammatory conditions which support a role for the CD39 and associated purinergic signalling pathways in inhibiting immune responses during chronic inflammation.

Increased numbers of $CD39^+$ cells that may provide anti-inflammatory functions have been isolated in inflammatory sites such as joints of arthritic patients [14], bronchoalveolar lavage from COPD patients [15], and lamina propria of patients with Crohn's disease [16]. Circulating antigen-reactive $CD4^+$ T-cells expressing CD39 were also increased in patients with active rather than latent tuberculosis [17]. There is growing evidence to suggest that CD39 inhibits IFNγ production. Notably depletion of $CD39^+$ Treg increased TB-specific antigen responses [18]. Furthermore, Bai *et al* demonstrated that $CD39^+$ $CD8^+$ T-cells inhibited IFNγ production by $CD39^-$ $CD8^+$ T-cells from healthy control samples [16].

Increased expression of CD39 has also been associated with other markers of T-cell exhaustion or dysfunction. This includes increased expression of PD-1 paralleling the decreased expression of CD28 and production of IFNγ [19]. In our patient cohort, we correlated increased $CD28^{null}$ T-cells in COPD and AECOPD with CMV-IgG titres [20]. Here increased expression of CD39 correlated with the loss of CD28 expression on $CD4^+$ T-cells ($r=-0.245$, $p=0.047$) and increased PD-1 expression ($r=0.278$, $p=0.024$). Within the Treg population, expression of CD39 also correlated with the expression of PD-1 ($r=0.882$, $p=0.004$). The data suggest that CD39 may promote immune exhaustion in patients with COPD and so contribute to the suppression of protective immune responses.

Our results build on the work by Lazar *et al* (2015) showing increased expression of CD39 on cells from bronchoalveolar lavage fluid and sputum of ex-smoking COPD patients than smokers and non-smoking controls [15]. They demonstrated that cigarette smoking induces the expression of CD39 on bronchoalveolar lavage cells and lung tissue, and proposed a compensatory upregulation of CD39 as a protective mechanism in cigarette smoke-induced lung damage. Our data suggests that the increased CD39 expression is not only limited to the lung environment but is also evident systemically, possibly as a compensatory response to chronic inflammation in COPD resulting in poorer T-cell responses. Blockade of CD39 may restore T-cell responses, but abrogation of CD39 in knockout mice promoted emphysema [15], and cigarette smoke can reduce CD39 expression in the lung, macrophages and vascular endothelial cells [21]. Therefore, further studies and refinements are required before CD39 is accepted as an appropriate theraupeutic target,.

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REFERENCES

1. Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 2013. **187**: 347-365.
2. Barnes PJ and Celli BR. Systemic manifestations and comorbidities of COPD. *Eur Respir J* 2009. **33**: 1165-1185.
3. Sethi S, Evans N, Grant BJ, and Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *N Engl J Med* 2002. **347**: 465-471.
4. Soler-Cataluna JJ, Martinez-Garcia MA, Roman Sanchez P, Salcedo E, Navarro M, and Ochando R. Severe acute exacerbations and mortality in patients with chronic obstructive pulmonary disease. *Thorax* 2005. **60**: 925-931.
5. Berenson CS, Garlipp MA, Grove LJ, Maloney J, and Sethi S. Impaired phagocytosis of nontypeable Haemophilus influenzae by human alveolar macrophages in chronic obstructive pulmonary disease. *J Infect Dis* 2006. **194**: 1375-1384.
6. King P, Ngui J, Oppedisano F, Robins-Browne R, Holmes P, and Holdsworth S. Effect of interferon gamma and CD40 ligation on intracellular monocyte survival of nontypeable Haemophilus influenzae. *APMIS* 2008. **116**: 1043-1049.
7. Taylor AE, Finney-Hayward TK, Quint JK, Thomas CM, Tudhope SJ, Wedzicha JA *et al.* Defective macrophage phagocytosis of bacteria in COPD. *Eur Respir J* 2010. **35**: 1039-1047.
8. Abe Y, Murphy TF, Sethi S, Faden HS, Dmochowski J, Harabuchi Y *et al.* Lymphocyte proliferative response to P6 of Haemophilus influenzae is associated with relative protection from exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002. **165**: 967-971.
9. Knobloch J, Schild K, Jungck D, Urban K, Muller K, Schweda EK *et al.* The T-helper cell type 1 immune response to gram-negative bacterial infections is impaired in COPD. *Am J Respir Crit Care Med* 2011. **183**: 204-214.

10. Antonioli L, Pacher P, Vizi ES, and Hasko G. CD39 and CD73 in immunity and inflammation. *Trends Mol Med* 2013. **19**: 355-367.

11. O'Garra A and Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004. **10**: 801-805.

12. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R *et al.* Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 2007. **110**: 1225-1232.

13. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 2007. **204**: 1257-1265.

14. Moncrieffe H, Nistala K, Kamhieh Y, Evans J, Eddaoudi A, Eaton S *et al.* High expression of the ectonucleotidase CD39 on T cells from the inflamed site identifies two distinct populations, one regulatory and one memory T cell population. *J Immunol* 2010. **185**: 134-143.

15. Lazar Z, Mullner N, Lucattelli M, Ayata CK, Cicko S, Yegutkin GG *et al.* NTPDase1/CD39 and aberrant purinergic signalling in the pathogenesis of COPD. *Eur Respir J* 2015.

16. Bai A, Moss A, Rothweiler S, Serena Longhi M, Wu Y, Junger WG *et al.* NADH oxidase-dependent CD39 expression by CD8(+) T cells modulates interferon gamma responses via generation of adenosine. *Nat Commun* 2015. **6**: 8819.

17. Kim K, Perera R, Tan DB, Fernandez S, Seddiki N, Waring J *et al.* Circulating mycobacterial-reactive CD4+ T cells with an immunosuppressive phenotype are higher in active tuberculosis than latent tuberculosis infection. *Tuberculosis (Edinb)* 2014. **94**: 494-501.

18. Chiacchio T, Casetti R, Butera O, Vanini V, Carrara S, Girardi E *et al.* Characterization of regulatory T cells identified as CD4(+)CD25(high)CD39(+) in patients with active tuberculosis. *Clin Exp Immunol* 2009. **156**: 463-470.

19. Gupta PK, Godec J, Wolski D, Adland E, Yates K, Pauken KE *et al.* CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. *PLoS Pathog* 2015. **11**: e1005177.

20. Tan DB, Amran FS, Teo TH, Price P, and Moodley YP. Levels of CMV-reactive antibodies correlate with the induction of CD28 T cells and systemic inflammation in chronic obstructive pulmonary disease (COPD). *Cell Mol Immunol* 2015.

21. Kratzer A, Salys J, Sévigny J, Zamora M, and Taraseviciene-Stewart L. Second hand smoke exposure impairs CD39 expression and function in the lung. *European Respiratory Journal* 2012. **40**.

FIGURE LEGENDS

Figure 1 – Gating strategies used to identify CD39$^+$ T-cell subsets: (A) Singlets were gated based on the expression of forward scatter-area (FCS-A) and FCS-Height. (B) Dead cells were then excluded and (C) lymphocytes were gated based on the expression of side scatter (SSC)-A and FSC-A. (D) CD4$^+$ or (E) CD8$^+$ T-cells were identified by co-expression of CD3 with CD4 or CD8 respectively. (F) Co-expression of CD39 and Foxp3 from CD4$^+$ T-cells.

Figure 2 – Increased plasma biomarkers of inflammation but decreased production of IFNγ by PBMC in AECOPD patients. A) CRP, B) IL-6 and C) sTNFR in plasma and levels of (D) IFNγ, (E) TNFα, (F) IL-6, (G), IL-17 and (H) IL-10 in supernatants of PBMC+SEB cultures were measured by ELISA. ***p<0.001, **p<0.01, *p<0.05, Mann-Whitney t-test.

Figure 3 – Frequencies of CD39 expressing T-cells were elevated in AECOPD patients and associate with decreased production of IFNγ in response to SEB. (A) Proportion of CD39$^+$ cells (as a % of parent population). (B-E) Correlation of IFNγ level in supernatants with proportion of CD39-expressing T-cell subsets. ***p<0.001, **p<0.01, *p<0.05, Mann-Whitney t-test.

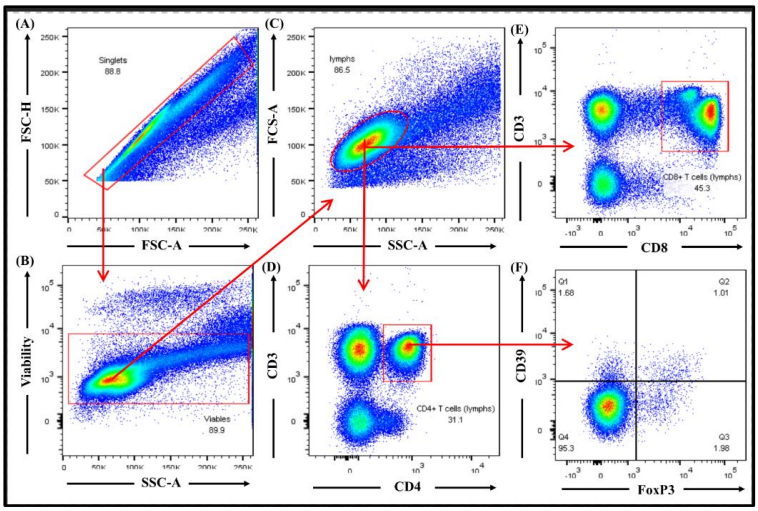

Figure 1

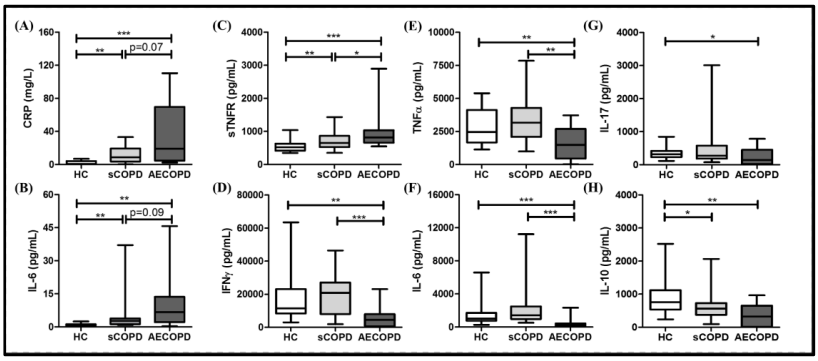

Figure 2

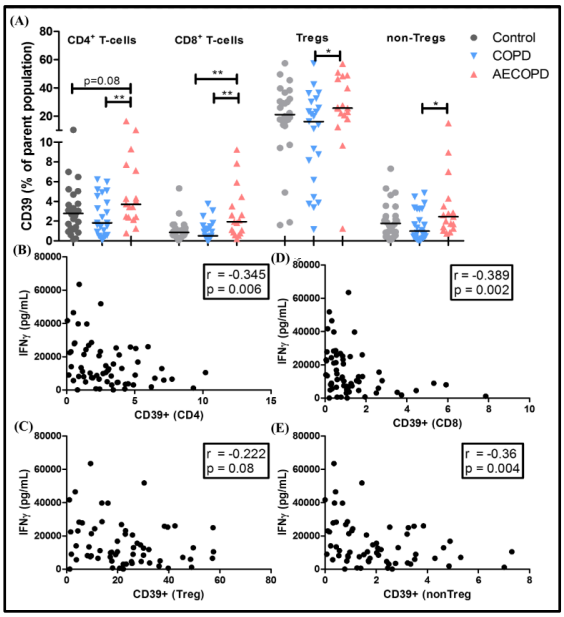

Figure 3