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CD81 and CD48 show different expression on blood eosinophils in systemic sclerosis –

new markers for disease and for pulmonary inflammation?

Article

Short title: CD48 and CD81 on blood eosinophils in SSc

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Abstract

Objective: In systemic sclerosis (SSc) related interstitial lung disease, elevated eosinophil

counts in bronchoalveolar lavage are associated with worse outcome. We hypothesize that

eosinophils are activated in the peripheral circulation, thereby increasing their recruitment to

affected tissues and contributing to inflammation and fibrosis. The aim of this study was to

characterize the blood eosinophils in SSc patients.

Methods: Expressions of surface markers CD11b, CD44, CD48, CD54, CD69, CD81 and

HLA-DR on CD16^{low}CD9^{high} expressing eosinophils were measured by flow cytometry in

whole blood from SSc patients (n = 32) and controls (n = 11).

Results: Expression of CD54, CD69 and HLA-DR were undetectable in all groups. CD44

and CD11b expression levels were similar between groups. CD81 expression was lower in

patients compared to controls independent of disease duration (p = 0.001). CD48 expression

was increased in patients with a short disease duration (< 2 years) compared to both controls

(p = 0.042) and patients with longer disease duration (p = 0.027). In patients with short

disease duration, increased CD48 expression was associated with alveolar inflammation as

measured by an increased concentration of alveolar nitric oxide (r = 0.76, p = 0.003).

Conclusion: Blood eosinophils change phenotype during disease evolution in SSc, and CD48

expression may be used as a biomarker for pulmonary inflammation.

Keywords: Systemic sclerosis; Eosinophils; CD48; CD81; CANO

Abbreviations: CaNO, concentration of alveolar nitric oxide; dcSSc, diffuse cutaneous SSc;

ILD, interstitial lung disease; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis

Introduction

In systemic sclerosis (SSc), interstitial lung disease (ILD) with fibrosis development is one of the major causes of death [1]. Inflammation may precede and contribute to development of pulmonary fibrosis and can be detected as increase in alveolar nitric oxide concentration (CaNO) [1]. Increased eosinophil counts in bronchoalveolar lavage have been associated with extent of pulmonary fibrosis [2] and with outgrowth of fibroblasts with altered phenotype from bronchoalveolar lavage fluid that may contribute to development of pulmonary fibrosis [3]. Also, increased eosinophil counts in bronchoalveolar lavage have been associated with increased mortality in SSc related ILD [4]. Eosinophils may thus play an important role in the inflammatory process and development of ILD in SSc that needs to be further elucidated.

CD81 belongs to the transmembrane 4 superfamily and is expressed on eosinophils. Data on CD81 expression on eosinophils are scarce. Increased levels of CD81 on eosinophils have been shown upon activation with interleukin-3 and GM-CSF [5], however reduced levels of CD81 was reported in another study on bronchial asthma after treatment with nedocromil [6].

CD48, or Blast-1, is a glycosyl-phosphatidyl-inositol-anchored protein that is expressed on several hematopoietic cells [7], and has been shown to interact with the immune receptors CD2 and CD244. CD48 can act as adhesion protein and has also co-stimulatory properties. CD48 has recently been linked to eosinophil biology in the context of experimental asthma [8] and allergic airway inflammation [9]. Ligation of CD48 resulted in eosinophil degranulation.

Systemic inflammation is present in SSc at low grade [10] and increased serum levels of eosinophilic major basic protein have been described in early SSc [11]. We hypothesized

therefore that eosinophils are activated in peripheral circulation facilitating their recruitment to the lung and that this is reflected in differentially expressed activation markers.

Materials and methods

Subjects

From July 2007 to June 2009, 32 consecutive SSc patients with a median (IQR) age of 63 (52 to 68) years and a median (IQR) disease duration of 2.75 (0.87 to 5.56) years were investigated. Disease on-set was defined as first non-Raynaud's symptom on-set. All 32 patients fulfilled the 2013 classification criteria for SSc [12]. Twenty-six patients (23 female, 3 men) had limited cutaneous SSc (lcSSc) and six patients (5 female, 1 man) had skin involvement proximal to elbow or knee and were classified as diffuse cutaneous SSc (dcSSc). None of the patients had received immunosuppressive treatment within one year prior to evaluation or at the evaluation. None of the patients were treated with prednisolone or inhalation steroids at evaluation. One subject was a current smoker and thirteen patients were ex-smokers. Eleven age-matched healthy individuals (all female) with a median (IQR) age of 57 (55 to 64) years were enrolled as control subjects. The Regional Ethics Board in Lund, Sweden, approved the study, and written informed consent was obtained from all patients.

Clinical assessment

All clinical and laboratory data from each patient reported in this study were obtained within one week of investigation. Patients underwent clinical and laboratory examinations as previously described [1, 13]. Disease on-set was defined as the first non Raynaud's manifestations.

Flow cytometry

Whole blood was collected in EDTA tubes and analysed the same day. Whole blood samples were stained for 10 minutes. Fluorescein isothiocyanate (FITC)-labelled anti-CD16 and peridine-chlorophyll protein Cy5.5 (PerCP-Cy5.5)-labelled anti-CD9 antibodies, both of IgG1 isotype (clone 3G8 and M-L13 respectively; BD Bioscience, San Jose, CA) were used for identification of eosinophil granulocytes. We selected a panel of activation marker that had previously been applied in studies of eosinophils, as reviewed in [14]. Surface molecules were assessed using phycoerythrin-labelled antibodies. Monoclonal antibodies specific for CD11b, CD44, CD48, CD69 and CD81 were of IgG1 isotype (clone ICRF44, 515, TÜ145, FN50 and JS-81, respectively; BD Bioscience) and monoclonal antibodies against CD54 and HLA-DR were of IgG2 isotype (clone HA58 and L243 respectively; BD Bioscience). Unspecific isotype-matched antibodies (IgG1 clone X40 and IgG2 clone X39; BD Bioscience) were used as controls. Fifteen microliter undiluted antibody solution of each antibody was added to 50 microliter whole blood and incubated for 10 minutes at room temperature. Cells were fixated and red blood cells were lysed in TQ-Prep (Beckman Coulter, Brea, CA) before acquisition of data. Eosinophils were identified as granulocytes (Figure 1A; population A) that stained low for CD16 (Figure 1B; population B) and high for CD9 (Figure 1C; population C2). Data acquisition was stopped after 1500 events were attained in the CD16^{low}/CD9^{high} gate or after 10 minutes. Analysis was performed on an Epics XL MCL flow cytometer (Beckman Coulter) using the CXP Software for acquisition and analysis. Data are expressed as median fluorescence intensity (MFI). The MFI for the isotype controls were median (range) 0.103 (0.103 - 0.195).

Cell sorting and light microscope analysis

To ensure that the CD16^{low}CD9^{high} granulocytes were eosinophils, five additional SSc patients were analysed for the expression of the eosinophil markers CD193 (CCR-3) and Siglec-8 on a

FACSCanto II using the DIVA 6.3 software (Becton Dickinson, BD, New York, NY, USA) and similar gating, Supplementary Figure. The following antibodies were used in this analysis: CD9-FITC, Siglec 8-PE, CD14-PerCP-Cy5.5, CD16-APC-H7, CD193-BD Horizon v500 all from BD bioscience. Two of these samples were subjected to cell sorting on a FACSAria II instrument and the CD16^{low}/CD9^{high} population were sorted directly on glass slides and studied under microscope.

Statistics

The statistical analyses were performed using STATISTICA v.12 (StatSoft, Tulsa, OK). Data are depicted as median and interquartile range (IQR). Kruskal–Wallis test was applied before comparisons between groups were made with Mann-Whitney test. Fisher's two-tailed exact test was used to compare frequencies. Spearman's test (r) was used to estimate correlations. Probability values (p) were considered significant when < 0.05.

Results

Patient characteristics

The demographic data of the patients are shown in Table 1. Patients disease duration was estimated from non-Raynaud's symptom on-set. Since the eosinophil counts suggested a disease duration dependent dynamic development, patients were divided into two populations based on disease duration: patients with a disease duration of less than 2 years and patients with a longer disease duration, i.e. 2 years and more.

Flow cytometric identification of eosinophil granulocytes in peripheral blood

The study was designed to analyse activity markers on CD16^{low}CD9^{high} eosinophils in peripheral whole blood, as previously described by others [15]. Additional analyses showed that the CD16^{low}CD9^{high} cells in peripheral blood expressed high levels of CD193 (CCR-3) and Siglec-8 and were therefore likely to reflected eosinophil granulocytes (Supplementary Figure). This was further confirmed by sorting out this population and examination of the cells under the microscope.

Eosinophil counts in peripheral blood

Patients with SSc had a tendency towards high count of eosinophils compared to the control individuals (p = 0.052). However, the eosinophil count increased with disease duration (r = 0.55, p = 0.001). While SSc patients with short disease duration had similar amount of eosinophils (median [IQR]) as the control individuals, (669 cells [280 to 750] versus 730 cells [371 to 1059]; p = n.s.), SSc patients with longer disease duration had significantly higher eosinophil counts (1500 cells [1222 to 1500]) compared to both control individuals and patients with short disease duration, p < 0.001 and p = 0.001 respectively. This finding was supported by standard differential cell counts that also showed that eosinophils were more abundant in SSc patients with longer disease duration than in patients with early disease (Table 1).

Surface expression of activation marker of eosinophil

Median fluorescence intensities of CD54, CD69 and HLA-DR were not statistically different from MFIs of the isotype control antibodies (data not shown). These markers were therefore

considered as not expressed and excluded from further analysis. The MFIs of the markers CD11b, CD44, CD48 and CD81 on eosinophils are shown in Figure 2.

Surface levels of CD81 were reduced in patients with SSc compared to control individuals, both as group (2.60 [2.22 to 3.23] versus 4.08 [2.85 to 4.92]; p = 0.001) and when the patients were divided into subgroups by disease duration, Figure 2 (lower right). Patients with early SSc had higher surface levels of CD48 compared to SSc patients with longer disease duration and healthy controls, p = 0.027 and p = 0.042 respectively (Figure 2, lower left). The surface levels of CD11b and CD44 in SSc patients did not differ from controls.

Association between Eosinophil surface marker expression and clinical data

Surface expression of the different activity markers was related to clinical and immunological data. Expression levels of the activation markers did not differ between limited cutaneous SSc or diffuse cutaneous SSc and was not related to modified Rodnan skin score. None of the activity markers correlated to C-reactive protein levels or to the erythrocyte sedimentation rate.

Association between eosinophil surface marker CD48 expression and auto-antibody state. Surface expression of the different activity markers was related to auto-antibody state. Patients were divided into groups with auto-antibody specificities of relevance for systemic sclerosis, i.e. anti-centromer (ACA), anti-RNA polymerase III (ARA), anti-DNA topoisomerase I (ATA, Scl-70) and anti-U1 ribonucleoprotein (RNP) antibodies; and into the remaing patients. Among the 4 eosinophil surface markers, CD48 was the only one that was differential expressed between the 5 groups when all patients were analysed (KW: p = 0.040;

Supplementary Figure 2) and when the patients with short diseases duration were analyses solely (KW: p = 0.053). Three patients with anti-RNP antibodies and short disease duration had the highest expression levels of CD48 on their eosinophils and accounted for this difference.

Association between CD48 surface expression, alveolar nitric oxide concentration (CANO) and pulmonary function test

In patients with early SSc, eosinophil surface levels of CD48 correlated positively with CaNO, (r = 0.76, p = 0.003; Figure 3), and inversely with vital capacity (r = -0.59, p = 0.027). These correlations remained also significant when patients were included with a disease duration up to 3 years. Thus, increased eosinophil surface levels of CD48 were correlated with increased CaNO (r = 0.71, p = 0.002, n = 16) and with decreased vital capacity (r = -0.68, p = 0.003, n = 17). When including all patients the eosinophil surface levels of CD48 were no longer correlated with CaNO levels (r = 0.19, p = 0.332, n = 30). Still, increased CD48 surface levels on eosinophils had a tendency to correlate with decreased vital capacity (r = -0.34, p = 0.061, n = 32). Neither expression levels of CD48 nor CD81 correlated with carbon monoxide diffusion capacity or radiologic extent of fibrosis.

Eosinophil surface marker CD11b expression and smoking

Patients with short disease duration included 4 ex-smoker, and patients with longer disease duration included one current smoker and 9 ex-smokers. The distribution of the smoker and ex-smoker was not significantly different between the groups (p = 0.182). For the whole patient group the comparison of the expression levels of the four surface markers on

peripheral eosinophils showed a tendency towards a lower CD11b expression in the smoker + ex-smoker group compared to never smoker (2.60 [2.22 to 3.23] versus 4.08 [2.85 to 4.92]; p = 0.054). In patients with early disease ex-smokers had significantly lower CD11b levels compared to never smokers (2.74 [1.92 to 3.56] versus 5.56 [4.83 to 6.54]; p = 0.007). In patients with longer disease duration smokers grouped together with ex-smokers did not differ from never smokers regarding expression of CD11b. We could not identify any link between smoking and the expression of CD48 and CD81 or the analysis of CANO. Smoking was therefore not considered to impact the presented results.

Discussion

This study shows that surface expression of activation marker on peripheral blood eosinophils varies between patients with disease duration of less than 2 years and patients with longer disease duration. Biomarkers are sought after to follow effects of early intervention. The cut-off was therefore set to 2 years, since this cut-off is commonly used in clinical trials of treatments regimes for the disease. Also, the initial study of eosinophils in SSc by Gustafsson et al [16] suggests a role for eosinophils early during disease pathogenesis. The data suggest a disease duration dependent dynamic evolution of the expression of the surface marker CD81 and CD48 on eosinophils in peripheral whole blood from patients with SSc.

Surface levels of CD81 are lower on eosinophils of SSc patients compared to healthy controls. Knowledge about the function of CD81 in eosinophil biology is very limited. It is only known that anti-CD81 antibodies reduce expression of the adhesion molecule CD62L (L-selectin) on eosinophils in vitro. CD81 is also a known marker for exosomes [17], but CD81 may also inhibit degranulation in mast cells [18]. We hypothesise that reduced CD81 surface levels may reflect degranulation of eosinophils. This degranulation may lead to an

increase of eosinophilic major basic protein in serum of SSc patients with early disease, as shown by Cox et al [11]. In the possible case that CD81 may prevent degranulation also in eosinophils, low CD81 surface expression on eosinophils seen in our study suggests that degranulation would likely occur. Circulating microparticles from other cell types are present in SSc. Whether or not eosinophils degranulate or shed microparticles in SSc needs experimental studies for confirmation. The reduced eosinophil CD81 surface levels also persisted in the group of patients with longer disease duration. Eosinophils appeared therefore to be affected not only early in the disease development but also after longer disease duration.

Patients with longer disease duration had also higher eosinophil counts than patients with short disease duration and control individuals. It is not known whether or not eosinophils are continuously mobilised from the bone marrow during disease development in SSc but remain in the peripheral circulation for a longer period of time, or if eosinophil mobilisation increases as disease duration progresses. During early disease, however, eosinophils appear to home to peripheral tissues to a greater extent than in later disease stages, as described by Gustafsson et al [16].

CD48 surface levels were increased on peripheral blood eosinophils in patients and correlated with CaNO in early SSc. Hence, eosinophils might contribute to alveolar inflammation as reflected by CaNO. Although CaNO is increased in patients with early SSc independently of radiological findings of SSc related interstitial lung disease [1], it is suggested by other that increased baseline CaNO may predict deterioration of pulmonary function in SSc [19]. Thus, eosinophil activation, as reflected by increased CD48 surface expression, and increased CaNO may reflect an early stage of pulmonary inflammation in SSc. Three patients with U-1 RNP antibodies had the highest CaNO values. Although pulmonary fibrosis is not the

foremost complication that is associated with U-1 RNP antibody profile in SSc, a summary by Virgina Steen shows that severe pulmonary fibrosis was present in 22 percent of patient with U1-RNP antibodies as compared to 26 percent of patients with ARA [20]. However, the mechanism for developing pulmonary fibrosis may differ between patients with different auto-antibodies profiles. The increased CD48 surface expression on eosinophils may reflect a subpopulation of patients with SSc and pulmonary involvement. Yet, further studies are warranted to confirm these measures as indicator for clinical significant pulmonary involvement.

The role for CD48 in the pathogenesis of SSc has not yet been investigated. CD48 can act as an adhesion molecule [7] and may lead to homing of eosinophils in peripheral tissue such as the lungs. Here, we could not find any relation between CD48 expression and extent of pulmonary fibrosis. Most of the patients in this study had mild disease and only two patients had extensive pulmonary fibrosis. The invers correlation of CD48 expression with vital capacity may suggest, however, that peripheral eosinophil activation could be related to pulmonary pathology. We have shown previously, that increased cell counts of eosinophils in bronchoalveolar lavage fluid are related to increased fibrosis on high resolution computer tomography scans [2]. Importantly, Goh et al identified an increased mortality in SSc associated ILD in patients with increased eosinophil counts in bronchoalveolar lavage [21].

A limitation of the present study is the small cohort of patients with SSc. However, immediate flow cytometer analysis' were required that necessitated a single centre set-up. Moreover, the requirement of patients without treatment with steroids or immunosuppressive agents in order to analyse the innate expression of the surface molecules in the disease stages reduced the number of patients with this rare disease eligible for inclusion. The results are therefore to be

our findings suggest a dynamic expression of the eosinophils activation marker that is dependent on disease duration. However, the cross sectional design of our study does not allow us to draw far-reaching conclusion on the connection between eosinophil activation marker expression and individual disease progression or eventual response to treatment. Also, the low amount of patients with severe pulmonary fibrosis limits further conclusions regarding whether or not eosinophils activation markers are reliable marker of severe pulmonary involvement.

In conclusion, we have detected that the activation markers CD81 and CD48 are differentially expressed on peripheral blood eosinophils in SSc patients with different disease durations. Reduced CD81 expression may reflect presence of the disease independent of the disease duration. High CD48 expression may be associated with pulmonary inflammation early in the disease development. Longitudinal studies are warranted to examine the utility of analysing these cell surface markers on eosinophils, or combinations thereof, as biomarker for disease, activity or prognosis.

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Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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Table 1. Demographic data for systemic sclerosis patients with short (< 2 years) or long disease duration (≥ 2 years)

	SSc < 2 years (n = 14)	$SSc \ge 2$ years $(n = 18)$
	number/number	number/number
Female/male	13/1	15/3
dcSSc/lcSSc	5/9 *	1/17
ANA/ATA/ACA	13/2/4 *	18/1/13
Pulmonary fibrosis	8/6/0	10/6/2
(extent: $0\% / \le 20\% / > 20\%$)		
	medians (IQRs)	medians (IQRs)
Age at exam (yrs.)	61 (49 to 68)	64 (54 to 67)
Disease duration (yrs.)	0.79 (0.5 to 1.0)	5.23 (3.42 to 18)
mRss (points)	5.5 (2 to 10)	3.5 (2 to 8)
VC (% p)	91 (84 to 95)	95 (81 to 108)
DLCO (% p)	76 (65 to 86)	64 (60 to 95)
FEV1 (% p)	89 (79 to 96)	93 (86 to 99)
CaNO (ppb)	3.68 (2.94 to 4.23)	3.4 (2.11 to 5.08)
Eosinophils (10 ⁹ cells/L)	$0.1 (0.0 \text{ to } 0.2)^*$	0.2 (0.1 to 0.2)
ESR (mm/h)	14 (12 to 18)	13 (9 to 18)
CRP (mg/L)	2.9 (1.3 to 3.8)	1.8 (0.73 to 4.6)
IgG (g/L)	10 (8.5 to 14.2)	8.8 (8.0 to 10.4)
IgA (g/L)	2.9 (2.6 to 4.1) **	1.85 (1.2 to 2.5)
IgM (g/L)	1.05 (0.76 to 1.3)	1.4 (0.72 to 1.8)

Data are shown as numbers or medians (IQRs). ACA, anti-centromer antibodies; ANA, anti-nuclear antibodies; ATA, anti-topoisomeras-1 antibodies; CANO, concentration of alveolar

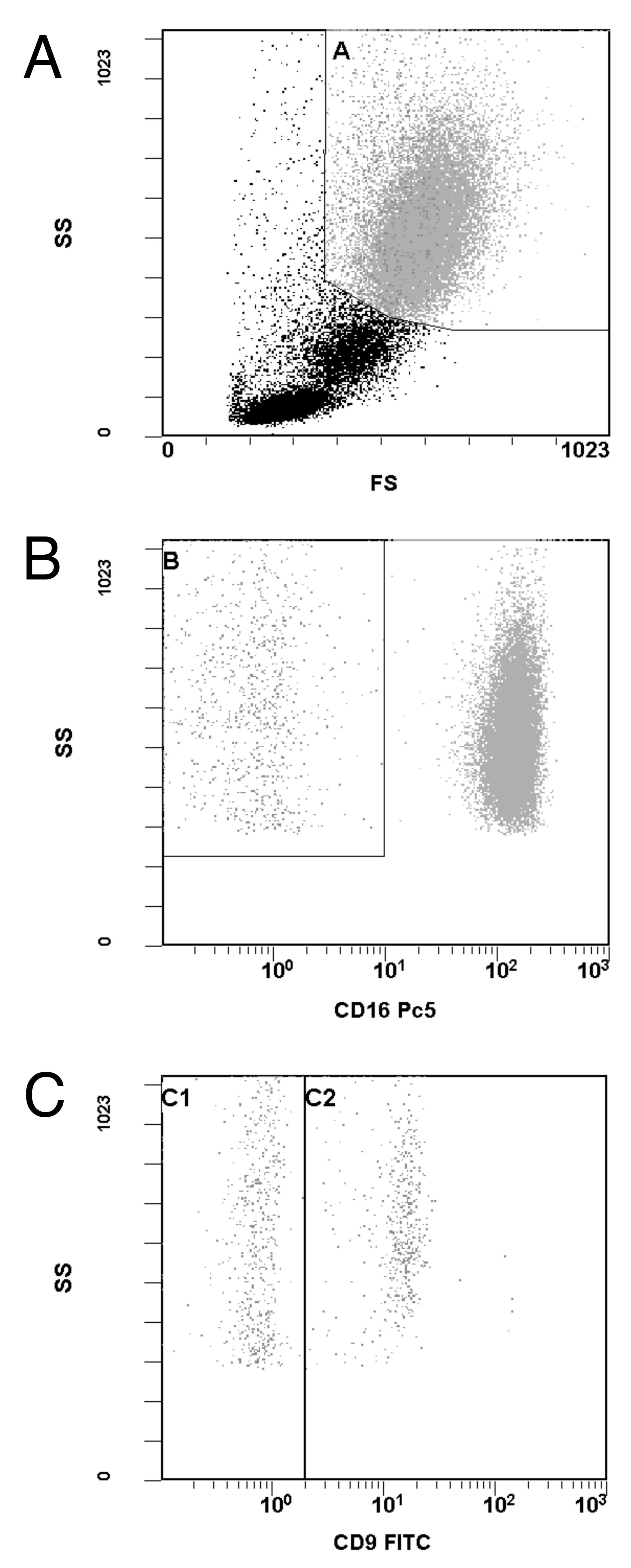
nitric oxide; CRP = C-reactive protein; dcSSc, diffuse cutanous SSc; DLCO, diffusing capacity for carbon monoxide; ESR, erythrocyte sedimentation rate; FEV1, forced expiratory volume in 1 second; Ig, immunoglobulin; ISSc, limited SSc; lcSSc, limited cutanous SSc; mRss, modified Rodnan skin score; ppb, parts per billion; % p, percent of predicted; VC, vital capacity. $^*p < 0.05$, $^{**}p < 0.01$.


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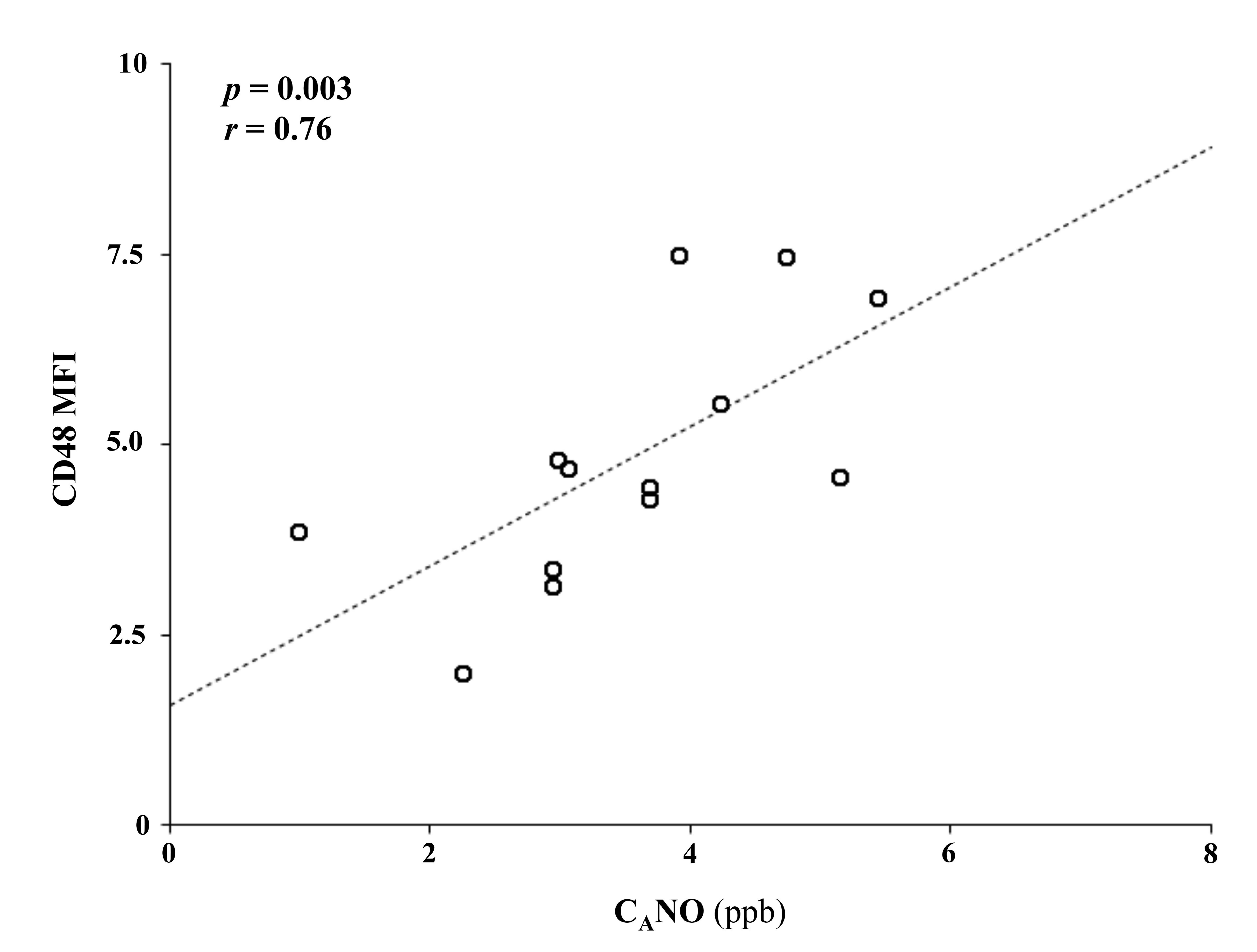
Figure 1. Flow cytometry gating for analysis of peripheral blood samples is shown. (A) Granulocytes were gated in forward and side scatter (population A). (B) In the second window the granulocytes (population A) were separated into CD16^{low} granulocytes (population B) and CD16^{high} neutrophils. (C) CD16^{low} granulocytes (population B) were further analysed for expression of CD9. Median fluorescence intensities (MFI) of the phycoerythrin-labelled activation markers were analysed within the CD16^{low}CD9^{high} gate (population C2) that were regarded as eosinophils. FS = forward scatter, SS = side scatter, Pc5 = Peridine-chlorophyll protein Cy5.5, FITC = fluorescein isothiocyanate.

Figure 2. (A) MFI of activity marker on CD16^{low}CD9^{high} blood eosinophils are shown for controls (n = 11) and systemic sclerosis patients with short (< 2 years, n = 14) or long disease duration (≥ 2 years, n = 18). Kruskal Wallis (KW) analysis shows significant differences between the groups for CD48 and CD81 expression. Boxes indicate median and interquartile range.

Figure 3. CD48 MFI on CD16^{low}CD9^{high} blood eosinophils is positively correlated to alveolar nitric oxide concentration (C_ANO) in patients with early systemic sclerosis. Depicted are SSc patients with a short disease duration (< 2 years) that had done the C_ANO analysis (n = 13).







Supplement to:

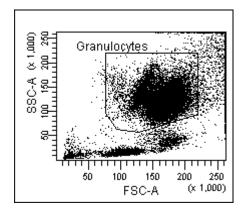
CD81 and CD48 show different expression on blood eosinophils in systemic sclerosis – new markers for disease and for pulmonary inflammation?

Supplementary Figure 1.

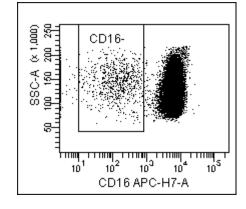
Flow cytometry gating is shown for analysis of eosinophil marker CCR3 (CD193) and Siglec-8 on peripheral blood samples of five additional patients. (**A**) Granulocytes were gated in forward and side scatter (population Granulocytes). (**B**) In the second window the granulocytes (population Granulocytes) were separated into CD16^{low} granulocytes (population CD16-) and CD16^{high} neutrophils. (**C**) CD16^{low} granulocytes (population CD16-) were further analysed for expression of CD9^{high} (population CD16-/CD9+). (**D**) CD16^{low}CD9^{high} expressing eosinophils were further analysed for expression of eosinophil marker CD193 (CCR-3) and Siglec-8.

Supplementary Figure 1.

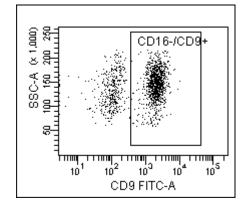




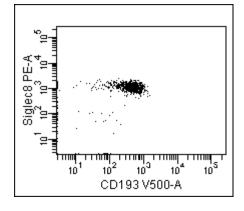
B)



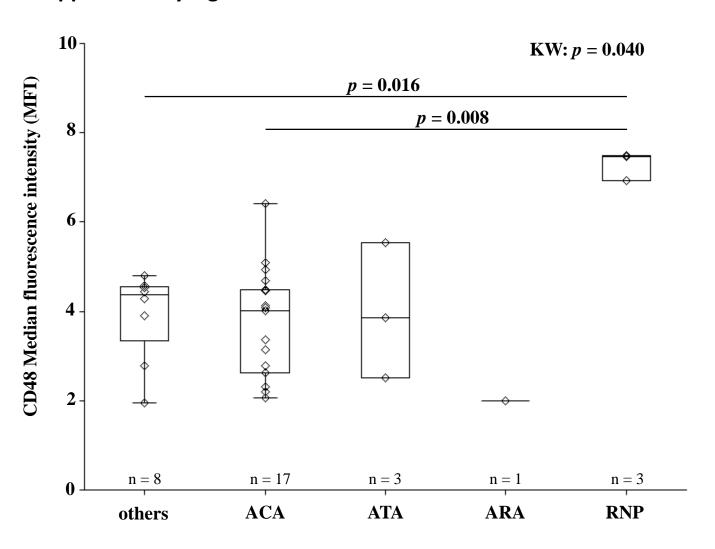
C)



D)



Supplementary Figure 2.



Supplementary Figure 2.

MFI of activity marker CD48 on CD16^{low}CD9^{high} blood eosinophils are shown for all systemic sclerosis patients. Patients are group by their antibody specificity. Kruskal Wallis (KW) analysis shows significant differences between the groups. Boxes indicate median and interquartile range. Others = this group consisted of patients with no antinuclear antibody (ANA, n=2) and patients with ANA but without the following antibody speceficities: ACA = anti-centromer antibodies, ARA = anti-RNA polymerase III antibody, ATA = anti- DNA topoisomerase I antibody (Scl-70), RNP = anti-U1 ribonucleoprotein antibody.