

Up-regulated dipeptidyl-peptidase IV (CD26) on monocytes was unaffected by effective DMARD treatment in early steroid and DMARD-naïve rheumatoid arthritis

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Abstract

Objectives

To study the CD26 density on monocytes and CD4+ T-lymphocytes in steroid and DMARD-naïve, early rheumatoid arthritis (RA) patients and to analyse for correlations with disease activity, including long-term radiographic progression.

Methods

Forty patients with active, early steroid and DMARD naïve RA (<6 months' duration) were randomised to treatment with methotrexate (MTX) versus MTX and cyclosporine A (CYA). Controls were 15 healthy age and gender matched subjects. Peripheral blood mononuclear cells were analysed for CD26 density by flow cytometry at baseline and after 52 weeks. Radiographic progression was scored by delta total Sharp-van der Heijde score (TSS) from 0 to 5 years.

Results

The density of CD26 on monocytes (CD3-CD14+) in RA was up-regulated compared to healthy controls ($p < 0.0001$) and remained unaffected by clinically effective DMARD treatment after 52 weeks. In anti-CCP positive RA patients ($n = 18$) baseline CD26 density on monocytes correlated to 5-year radiographic progression ($p = 0.008$, $r = 0.60$). The density of CD26 did not correlate to DAS28, the swollen or tender joint count or CRP-level at baseline or at year one. The CD26 density on CD4+ T-lymphocytes at week 0 was comparable to healthy controls ($p = 0.34$).

Conclusions

The up-regulated density of CD26 on monocytes in steroid and DMARD naïve active early RA was unaffected by 52 weeks of effective DMARD treatment and correlated to 5-year radiographic progression.

Key words

rheumatoid arthritis, CD26, monocytes, CD4+ T-lymphocytes

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Introduction

Dipeptidyl peptidase IV (DPP-IV; CD26) is a 110-kDa cell surface glycoprotein localised on the surface of T-lymphocytes and monocytes isolated from synovial fluid and peripheral blood in RA (1-8).

CD26 has been localised by immunohistochemistry to T-lymphocytes in the sublining region of synovium in rheumatoid arthritis (RA) (9). The activity of CD26 in synovial specimens has been reported both elevated and equal in RA patients compared to posttraumatic controls as well as osteoarthritis (OA) (1, 10), but the CD26 expression in mononuclear cells in synovial fluid (SF) has been reported lower than in OA (1). The soluble CD26 levels in serum from RA patients have been found both reduced and normal and to correlate with the number of swollen joints (11-13). Circulating auto-antibodies against CD26 in RA have been described at levels similar to healthy controls (12).

Several *in vitro* studies indicate that CD26 is involved in T-lymphocyte activation and proliferation (3, 14-17) and in changing the Th1/Th2 balance towards a Th1-reaction (18). *In vitro* CD26 has also been described to modulate migratory responses, possibly by truncation (inactivation) of chemokines, neuropeptides and cytokines (19-23).

Recent *in vivo* experimental data suggest that CD26 is protective against invasion of synovial fibroblast into cartilage (24). CD26 is widely distributed in inflamed and neoplastic tissue (25, 26), and most of the experimental and clinical data point towards an engagement of membrane bound CD26 as a pro-inflammatory molecule.

The objective of this study was to quantify the CD26 density on circulating monocytes and CD4⁺ T-lymphocytes in DMARD-naïve, early RA patients, and to analyse for correlations with disease activity, including long-term radiographic progression.

Patients and methods

Characterisation of patients and healthy controls

This study includes 40 patients with early active DMARD and steroid naïve

RA (disease duration <6 months) that fulfilled the American College of Rheumatology (ACR) 1987 revised criteria (27). They all participated in the CIMESTRA study and were enrolled at the department of Rheumatology Aarhus University Hospital (28). The baseline characteristics of the patients are listed in Table I. All patients received MTX and were randomised to double blinded treatment with cyclosporine A (CYA) or placebo. Oral steroid treatment was not allowed, but intra-articular steroid injection was performed in case of joint swelling, with a limitation of four joints and/or a total dose of 4 ml of betamethasone (160 mg) per visit. The escalation of MTX or MTX/CYA and the accumulated intraarticular betamethasone use have been previously described (28).

Disease activity was assessed in each patient every fortnight for the first 2 months and monthly thereafter. Disease activity assessment encompassed: the swollen and tender joint counts (40/40); 100 mm visual analogue scales (VAS) for doctor's and patient's global assessment of disease activity and patient's pain score and the HAQ-score (Stanford Health Assessment Questionnaire). Assessment of response to DMARD treatment was in accordance with the ACR criteria for improvement (29, 30). In the MTX/CYA group an increased number of anti-CCP positive patients were observed, 16 *versus* 8 patients, compared to the MTX group at inclusion, $p=0.02$ (chi-square test).

Conventional radiographs of the hands (posteroanterior and Nørgaard [31] projections), wrists (posteroanterior and lateral projections), and forefeet (anteroposterior view) were obtained at baseline and after 1 and 5 years. The radiographs were scored by an independent senior musculoskeletal radiologist (AaV) using the Sharp-van der Heijde scoring method (32). He was blinded to treatment group assignment, but not to the chronologic order of the images. The primary radiographic end point was changed in total Sharp-van der Heijde score (TSS) from baseline.

Cell separation and flow cytometry

The peripheral blood mononuclear cells (PBMC) were isolated from heparinised

Table I. Baseline demographic, clinical and laboratory characteristics of patients.

Flow cytometry	RA patients n=40
Age (years)	50.5 (24.5–72.6)
Female sex n (%)	26 (65)
Never smokers n (%)	12 (30)
Disease duration (weeks)	12.7 (6.4–23.8)
IgM-RF positive baseline n (%)	31 (78)
Anti-CCP positive baseline n (%)	24 (60)
TJC (0–40)	12.5 (1–38)
SJC (0–40)	8 (3–37)
Doctor global (0–100 mm)	37 (3–78)
Pain (0–100 mm)	40 (2–95)
Patient global (0–100 mm)	42 (3–95)
Serum Crp (mg/L)	18.8 (0.0–253.1)
DAS 28	5.25 (2.57–8.04)
HAQ score (0–3)	1.00 (0–2.63)
Radiographic findings	
Erosive disease n (%)	26 (65)
Progression (delta TSS >0) n (%)	
Baseline - year 1	11 (27)
Baseline - year 5	30 (72)
Total sharp score (mean median)*	
Baseline	5.4 3.0
Year 1	6.4 4.0
Year 5	11.1 5.0
Delta TSS* (mean median)	
Baseline - year 1	0.8 0.0
Baseline - year 5	5.8 2.0

*Radiographic progression was scored by delta total Sharp-van der Heijde score (TSS)(32) from 0 to 5 years.

Values are number of patients n and percentage (%) or mean and median. Anti-CCP: antibodies to cyclic citrullinated peptide in serum.

venous blood immediately after sampling. The PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St Louis, MO, USA). PBMC's were collected from the interface. The cells were washed three times by centrifugation at 400 G for 15 min. in RPMI-1640 medium without L-glutamine (Pharmacia, Upsala Sweden), supplemented with penicillin (10.000 U/l) and streptomycin (10.000 µg/l) (Sigma-Aldrich, St Louis, MO, USA), counted and frozen slowly according to a standard freezing procedure, and stored at -140° C (33). Flow cytometry analysis was performed blinded to the clinical data. Viability was assessed by propidium iodide staining before fixation (1.0 mg/ml Sigma), 10 µl added to the cell-suspension and washed by gentle centrifugation before flow cytometry.

Phycoerythrin (PE) conjugated monoclonal mouse antibodies (MoAbs) against CD26 in combination with allo-phyco-cyamin (APC) conjugated MoAbs against CD14 and CD4 were used. Relevant fluorochrome conjugated isotype IgG's (MoAbs) were used as controls. Monoclonal antibodies were all purchased from Becton-Dickinson, Mountain view, CA, USA (B-D), Diatec, Oslo, Norway or R&D Systems, Abingdon, UK (R&D). Combinations of monoclonal antibodies and fluorescent conjugations were used as listed by producer and catalogue number: Vial 1: CD45-Fluorescein-iso-thiocyanat (FITC)(B-D 345808) in combination with CD14-PE (B-D 345755) and CD3-Peridin-chlorophyll (PERCP)(B-D 555341) and CD4-APC (Diatec 3023); Vial 2: CD26-PE (B-D 555437) and CD14-APC (Diatec 3113); Vial 3: CD26-PE (B-D 555437) and CD4-APC (Diatec 3023); Vial 4: IgG-FITC (Diatec 2011) and IgG-PE (Diatec 2012) and IgG-PERCP (B-D 555750) and CD14-APC (Diatec 3113); Vial 5: IgG-FITC (Diatec 2011) and IgG-PE (Diatec 2012) and CD3-PERCP (B-D 555341) and CD14-PE (Diatec 3113); Vial 6: IgG-FITC (Diatec 2011) and IgG-PE (Diatec 2012) and IgG-PERCP (B-D 555750) and CD4-APC (Diatec 3023).

Cells were incubated for 30 min. at 4°C, at antibody concentrations recommended by the manufacturers, followed by washing and fixation in 0.1 M paraformaldehyd phosphate buffered saline (PBS) pH 7.4. A FACS Calibur flow cytometer ([B-D], Mountain View, CA, USA) was used for flow cytometric assessment of phenotypic markers, acquiring at least 10000 events in a live forward scatter (FSC) gate/side scatter (SSC) gate. Three- or four-colour fluorescence was detected using log amplification without compensation for spectral overlap. Compensation for spectral overlap was performed using single staining and negative controls in the FlowJo software package, version 6.47 (TreeStar, San Carlos, CA, USA). Listmode gating of phenotypical subsets of PBMC was applied for specific analysis of CD26 density in FlowJo® software version 4.5.2 (Tree Star Inc, San Carlos, CA, USA). Specific fluo-

rescence intensity of positively stained cells was defined by subtraction of non-specific IgG-signals. Aspects of validation of the method used in the evaluation of the flow cytometry have been published (8, 34).

Flow cytometry at baseline (week 0) and week 52 was performed in each patient in one session of thawing, incubation and fixation of the cells to avoid day-to-day assay variation.

Ethical considerations

The protocol was approved by the Danish National Board of Health and the Danish National Committee on Biomedical Research Ethics (no. M-1959-98) and registered at www.clinicaltrials.gov (no. NCT00209859). The participants gave written consent according to the Declaration of Helsinki (35).

Statistics

The statistical analysis was performed blinded to data collection and laboratory analysis. The Mann-Whitney rank sum test and the Wilcoxon signed rank test were used to compare the two groups of data and to compare data at baseline (week 0) with data at week 52. Chi-square test was used to compare the number of anti-CCP positive patients at baseline between treatment groups and the Spearman correlation analysis was used to assess for correlations between parameters of flow cytometrical reproducibility as well as correlations between the ACR core parameters and the CD26 median fluorescence density on monocytes and CD4⁺ T-lymphocytes. Spearman correlation analysis was also performed to test possible correlation between the median CD26 density (fluorescence signal) and the negative control signal. Significance was defined at the 5% level. The 'R' software package (36) was used for statistical analysis which was performed by an independent statistician.

Results

Cell viability, flow cytometry background staining

Viability by propidium-iodid staining in a live FSC gate/side scatter SSC gate showed a median of 79.1% viable cells (range 31.4–96%, n=95 [15

Table II. CD26 density evaluated by flow cytometry in healthy controls and DMARD naïve rheumatoid arthritis patients at baseline.

	Healthy controls (n=15)	DMARD naïve early RA (n=40)
CD14 ⁺ CD26mfi	1.1 (0–2.7)	4.1 (0–8.95)*
CD4 ⁺ CD26 ⁺ mfi	129.2 (102.0–191.0)	145.4 (61.2–253.0)
CD4 ⁺ CD26 ⁺ %	85.8 (64.5–92.9)	83.9 (59.7–94.8)

Values listed as median and range. mfi: mean fluorescence intensity.

Before initiating DMARD treatment the density of CD26 on monocytes (CD3-CD14⁺) was up-regulated compared to healthy controls ($p < 0.0001$)*

The CD26 density on CD4⁺ T-lymphocytes at baseline was not up-regulated compared to healthy controls ($p = 0.34$).

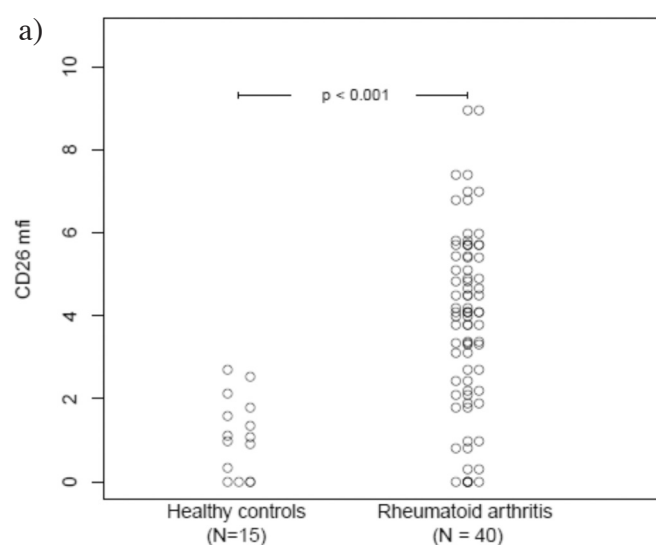
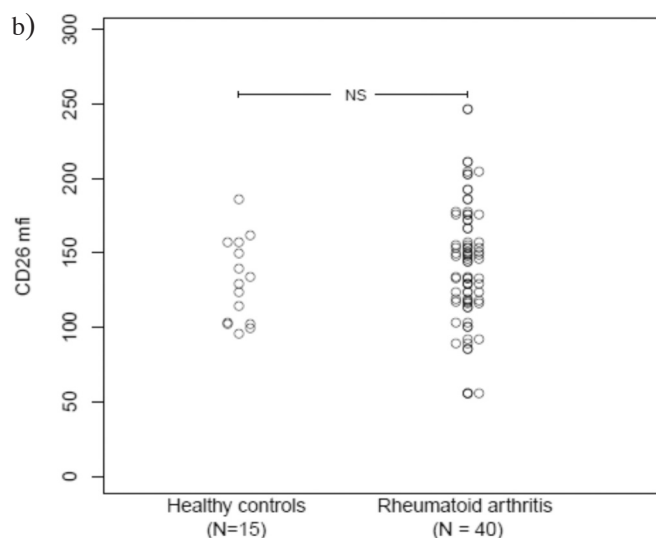


Fig. 1a. Increased density of CD26 on monocytes was observed in early DMARD-naïve RA patients * ($p < 0.001$).

1b. The density of CD26 on CD4⁺ T-lymphocytes in early DMARD-naïve RA patients equalled healthy controls * ($p = 0.34$).



healthy controls, 40 patients measured at week 0 and 52]) being propidium-iodid negative.

To evaluate whether the CD26 signal in CD45⁺CD14⁺ cells fluctuated with the background IgG signal in CD45⁺CD14⁺ and CD45⁺CD4⁺ cells or

not, a linear regression was performed with the CD26pe median signal as ordinate and the IgG signal as coordinate and no significant linear relation was observed ($r = 0.21$, $p = 0.06$; $r = 0.11$, $p = 0.14$). This indicates specific binding of the CD26 antibody to the cell surface

and that the variation of CD26 signals was not caused by spectral overlap from the co-stainings.

Evaluation of the monocytes

Compared to healthy controls, increased CD26 density was found in the 40 RA patients at baseline ($p = 0.00004$) (Table II and Fig. 1a). The increased CD26 density on monocytes was unaffected by 52 weeks of MTX or MTX/CYA-treatment ($p = 0.17$ and $p = 0.88$).

Using Spearman's test, no significant correlation was found between the ACR core parameters and the CD26pe median fluorescence density on monocytes before or one year after MTX and MTX/CYA treatment was started. No difference was observed between the MTX and MTX/CYA treated groups on the density of CD26 on monocytes before and after 52 weeks of treatment. In anti-CCP positive RA patients ($n = 24$, [16 patients from the MTX/CyA-group and 8 patients from the MTX/placebo-group]) we observed a significant correlation between baseline CD26 density on monocytes and 5-year radiographic progression ($p = 0.008$, $r = 0.60$) illustrated in Figure 2 (in 6 of the total 24 anti-CCP positive patients, the 5-year radiographic follow-up was not obtained).

In summary, the CD26 density on monocytes was increased both at week 0 and after 52 weeks of treatment compared to healthy controls (Table II and Fig. 1a). No significant correlation was observed between the CD26 density on monocytes and the CRP-level, number of tender or swollen joints, or DAS28. The baseline CD26 density on monocytes in anti-CCP positive patients ($n = 18$) correlated to the 5-year radiographic progression (Fig. 2).

Evaluation of the CD4⁺ T-lymphocytes

The CD26 density found on the CD4⁺CD26⁺ T-lymphocytes in the 40 RA patients at baseline equalled the density found in healthy controls ($p = 0.34$) (Table II and Fig. 1b), and was unaffected during 52 weeks of MTX or MTX/CYA-treatment ($p = 0.26$ or $p = 0.09$).

No difference was observed between the MTX and MTX/CYA treated groups on the density of CD26 on CD4⁺ T-lym-

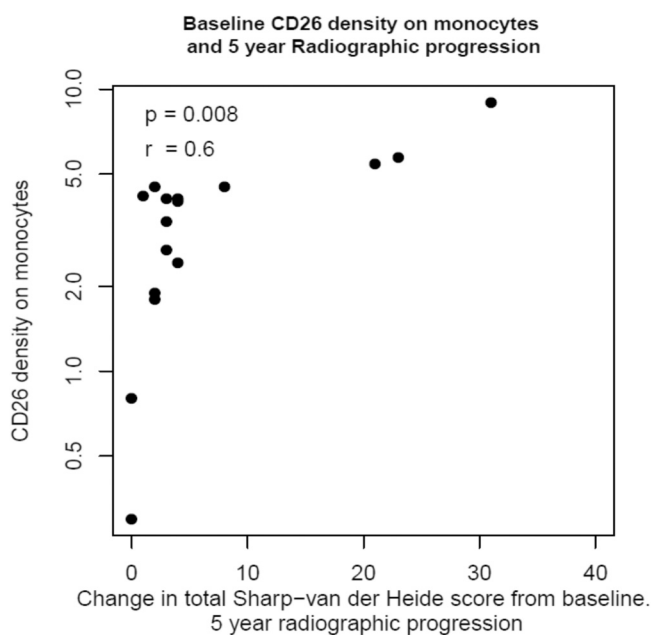


Fig. 2. Illustrating the correlation between median CD26 density on monocytes and the 5-year radiographic progression in anti-CCP positive RA patients.

phocytes at either week 0 or 52 ($p=0.11$ and 0.68) (Fig. 1b). Significant correlations were observed between the CD26 density on CD4⁺CD26⁺ T-lymphocytes and the CRP at baseline and week 52 (week 0: $p=0.008$, $r=0.41$; week 52: $p=0.03$, $r=0.36$) and the DAS28 at baseline, but not at week 52 (week 0: $p=0.015$, $r=0.38$; week 52: $p=0.06$, $r=0.32$).

After 52 weeks of treatment the percentage of CD4⁺CD26⁺ T-lymphocytes increased in the MTX/CYA group in contrast to the MTX group ($p=0.0005$ and $p=0.26$) (Table II). No difference was observed between the MTX and MTX/CYA treated groups on the percentage of CD4⁺CD26⁺ T-lymphocytes at either baseline or week 52 ($p=0.70$ and 0.46). No correlation was observed regarding CD26 density on CD4⁺ T-lymphocytes and radiographic progression.

Discussion

Our main findings were that the increased density of CD26 on circulating monocytes remained up-regulated after one year of clinically effective DMARD treatment in early steroid and DMARD-naïve RA patients. The significant increase of CD26 density on circulating monocytes is in keeping with our earlier report of increased CD26 monocytes from chronic RA patients (8). The increased density of CD26 on circulating monocytes correlated to ra-

diographic progression over 5 years in the anti CCP positive early RA patients ($n=18$). The interpretation of this observation should be cautious due to the limited number of patients. Recently, an augmentation in soluble CD26 levels during anti-TNF treatment in RA has been described (37). A specific inhibitor of CD26 can modulate the production of IL-1 receptor antagonist, IL-6 and transforming growth factor β 1 in lipopolysaccharide *in vitro* stimulated monocyte cultures (38). Furthermore, soluble CD26 up-regulates CD86 on circulating monocytes (39). In good agreement with our results, those studies indicate a role for CD26 in monocyte activation.

The present study adds that the increased CD26 density on circulating monocytes, at baseline, was unaffected by 1 year of clinically effective MTX and MTX/CYA treatment in DMARD-naïve RA patients.

Previously, we reported a significant increase in CD26 density on CD4⁺ T-lymphocytes in chronic RA patients (8). In our 40 DMARD-naïve early RA patients, with disease duration of less than 6 months followed for one year, we did not observe increased CD26 density on CD4⁺ T-lymphocytes. This indicates that increased CD26 density on CD4⁺ T-lymphocytes is probably a late phenomenon in the immune response in patients with RA. To which extent disease duration, disease activ-

ity, treatment or specific pathogenetic mechanisms influence the late increase of the immune-response in RA remains to be clarified.

We consider our flow cytometric assessment to be valid since we found no correlation with the CD26 density and the background staining. We have recently described our flow cytometric evaluation as reproducible and reliable (8, 34).

Conclusion

In conclusion, this study adds that CD26 density is enhanced on monocytes not only in chronic RA patients, but also in DMARD naïve active RA patients with disease duration less than 6 months. Our results support the hypothesis that CD26 on monocytes is a pro-inflammatory molecule involved in the pathogenesis in early RA.

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