Atherosclerosis in rheumatoid arthritis: associations between anti-cytomegalovirus IgG antibodies, CD4+CD28null T-cells, CD8+CD28null T-cells and intima-media thickness

B. Wahlin[1], A.E.R. Fasth[2], K. Karp[3], K. Lejon[4], V. Malmström[2], A. Rahbar[5], S. Wållberg-Jonsson[1†], A. Södergren[1,6]

[1]Department of Public Health and Clinical Medicine/Rheumatology, Umeå University, Sweden; [2]Rheumatology Division, Department of Medicine at Solna, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden; [3]Department of Surgical and Perioperative Sciences/Clinical Physiology, Umeå University, Sweden; [4]Department of Clinical Microbiology, Infection and Immunology, Umeå University, Sweden; [5]Division of Microbial Pathogens, BioClinicum, Department of Medicine at Solna, Karolinska Universtity Hospital, Karolinska Institutet, Stockholm, Sweden; [6]Wallenberg Centre for Molecular Medicine (WCMM), Umeå University, Sweden.

Abstract

Objective

Patients with rheumatoid arthritis (RA) have an accelerated progression of atherosclerosis. The aims of this study were to study the associations between subsets of T-cells, subclinical atherosclerosis assessed by intima-media thickness (IMT) and serological status for CMV in patients with RA.

Methods

Patients with new-onset RA (n=79), aged ≤60 years at diagnosis, were included in a prospective study of atherosclerosis. Controls matched for age and sex were also included (n=44). Ultrasound measurement of IMT in the common carotid artery was undertaken at inclusion (T0), after 1.5 years (T1.5) and after 11 years (T11). At T11, flow-cytometry analysis was undertaken to investigate subsets of T-cells. Serological analysis for CMV was undertaken from samples collected at T0.

Results

At T0, 66% of the patients and controls were CMV immunoglobulin G-positive. CMV-IgG positive patients had a significantly more rapid increase in IMT at T1.5, compared with controls and CMV-IgG negative patients. CMV-IgG positive patients had a significantly higher percentage of T-cells lacking CD28 (both $CD4^+CD28null$ and $CD8^+CD28null$ T-cells) than CMV-IgG negative patients. Increased levels of $CD4^+CD28null$ and $CD8^+CD28null$ T-cells were significantly associated with IMT at T11, adjusted for systolic blood pressure. CX3CR1 was expressed in $CD4^+$ and $CD8^+$ CD28null T-cells, but CX3CR1 per se was not associated with increased IMT.

Conclusion

Presence of CMV IgG-antibodies in patients with RA is associated with altered T-cell-populations and an increased burden of atherosclerosis. A possible protective effect of antiviral treatment in CMV-positive patients with new-onset RA should be considered.

Key words

rheumatoid arthritis, atherosclerosis, T-cells, CD28, cytomegalovirus

Bengt Wahlin, MD, PhD
Andreas E.R. Fasth, PhD
Kjell Karp, MD, PhD
Kristina Lejon, PhD
Vivianne Malmström, PhD
Afsar Rahbar, PhD
Solveig Wållberg-Jonsson, MD, PhD†
Anna Södergren, MD, PhD

†Solveig Wållberg-Jonsson died on May 2nd, 2019.

Please address correspondence to:
Bengt Wahlin,
Reumatologkliniken NUS,
90185 Umeå, Sweden.
E-mail: bengt.wahlin@umu.se

Received on February 10, 2020; accepted in revised form on June 1, 2020.

Funding: this study was funded by Västerbotten County Council, Umeå University, King Gustaf V's 80-year Foundation and the Swedish Rheumatism Association.

Competing interests: none declared.

Introduction

Morbidity and mortality due to cardiovascular disease are increased in patients with rheumatoid arthritis (RA), compared to the general population (1-3). This increase in cardiovascular disease reflects the fact that patients with RA have a higher burden of atherosclerosis (4, 5). The mechanisms causing increased atherosclerosis in RA are not fully understood, but the increase cannot be explained merely by traditional risk factors, nor by inflammation or other RA-specific factors (6).

Atherosclerosis is an inflammatory process, involving T-cells (7). To be activated, naïve T-cells need co-stimulation from the co-receptor CD28. Patients with RA have an altered T-cell population, where $CD4^+$ T-cell clones with cytotoxic features and signs of chronic activation are expanded and there is an increased number of $CD4^+$ T-cells lacking CD28 (CD28null T-cells) (8, 9). These cells are terminally differentiated and pro-inflammatory (10). Previous studies have shown increased numbers of $CD4^+$CD28null T-cells in patients with cardiovascular disease in the general population (11). In previous studies in patients with RA, $CD4^+$CD28null T-cells were associated with increased intima-media thickness (IMT) and coronary artery calcification (12, 13).

$CD8^+$CD28null T-cells have been less studied in atherosclerotic diseases, although $CD8^+$ T-cells are a prominent inflammatory cell type in advanced atherosclerotic lesions (14). However, altered $CD8^+$ T-cell-populations have been linked to atherosclerosis in the general population (15, 16).

Infection with human cytomegalovirus (CMV) is associated with increased numbers of CD28null T-cells in both patients with rheumatoid arthritis (17, 18) and in the general population (19-21). There are several studies where CMV-infection has been associated with atherosclerosis in the general population (22), but to the best of our knowledge, no such studies on patients with RA have been published.

In a previous study of patients with RA, CX3CR1 expression on the surface of $CD4^+$CD28null T-cells was associated with increased IMT (23). CX3CR1 is the receptor of fractalkine, a membrane-bound chemokine that is expressed on activated endothelium. Thus, the fractalkine-CX3CR1 interaction mediates leucocyte adhesion to the activated endothelium (24). Both the expression of CX3CR1 on the surface of T-cells and the expression of fractalkine on endothelial cells increase during CMV-infection (25).

This study aims to investigate possible associations between subsets of T-cells, CX3CR1 expression, serological status for CMV, and subclinical atherosclerosis assessed by IMT in a prospective cohort of patient with recent onset RA.

Methods

Patients

This study was undertaken as part of an ongoing prospective study of development of atherosclerosis in patients with early RA. The procedures have previously been described in detail (26). In short, patients diagnosed with RA (fulfilling the American College of Rheumatology criteria of 1987 (27)), less than 60 years of age and with symptoms of RA for less than one year at diagnosis, were invited to a prospective study of atherosclerosis (n=71). The first assessment at the start of the study is hereafter called T0. At follow-up 11 years after inclusion in the study (T11), 58 patients were re-examined. Due to a hardware crash, ultrasound examinations from seven patients were lost and IMT could not be analysed in those patients. One patient did not participate in the flow-cytometry analysis at T11, leaving 50 patients with complete data from T11. At T0, 44 controls without RA were included, of whom 29 were re-assessed at T11. The controls were matched for age and sex at T0. An unselected subgroup of 27 patients with RA, and their matched controls, were also examined 1.5 years after inclusion in the study (T1.5). Blood lipids were measured by routine methods at each hospital. At the study visits, blood pressure was measured. Cardiovascular risk factors were assessed from a patients survey studies and studies of patients' records. Descriptive data on the 50 patients and 29 controls included in

this study are presented in Table I. Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) 1858T allele, HLA-DRB1 shared epitope (SE) (defined as 0101/0401/0404/0405/0408), anti-CCP and Larsen score were assessed as previously described (28). The patients were treated for their RA according to clinical practice during the study period. The study was performed in line with the Helsinki declaration and approval was obtained from the regional Board of Ethics at Umeå University (Dnr 01-183). All participants gave their written informed consent.

Ultrasound

Ultrasound examination of the right common carotid artery was undertaken at T0, T1.5 and T11. The IMT was measured in an approximately 10 mm long part of the far arterial wall, proximal to the carotid bulb. Three measurements were done, and the mean value was used in further analyses. All ultrasound examinations were performed by the same experienced investigator. Details on ultrasound measurement have been described previously (26).

Flow-cytometry

At T11, T-cell populations were examined by flow-cytometry analysis. T-cells were identified by CD45 (common leukocyte antigen) and CD3 (T-cell marker). Further subsets were studied by detection of CD4, CD8, CD28, CD56 and CX3CR1.

For each individual, BD Heparin Vacutainer blood collecting was used. The blood was stored in room temperature up to 24h before further processing. To isolate mononuclear cells from whole blood, blood was diluted 1:1 in phosphate buffered saline (PBS) without calcium and magnesium (Life Technologies) and layered on Ficoll-Paque Plus (Fisher Scientific) and centrifuged 20min at 400g according to manufacturer's instructions. Mononuclear cells were resuspended in fluoresence activated cell sorter (FACS) buffer (PBS supplemented with 2% heat inactivated sterile filtered foetal calf serum (Fisher Scientific) and sodium azide [0.09 % (w/v)]). Cells were counted using Trypan Blue and $2x10^6$ mono-nuclear cells per individual were stained with antibodies against CD45 AF700 (HI30, BioLegend), CD8 BV421 (RPA-T8, BioLegend), CD3 PerCP (SP34-2, BD), CD4 AF488 (RPA-T4, BD), CD56 BV506 (NCAM16.2, BD),

Table I. Demographic data, cardiovascular risk factors and RA associated variables in 50 patients with RA and 29 controls matched for sex and age.

Demographic	RA (n=50)	Controls (n=29)
Age (years), mean (SD)	59 (9.7)	60 (10.7)
Female, n (%)	43 (86)	25 (81)
Cardiovascular risk factors		
Diabetes at T0, n (%)	0	0
Diabetes at T11, n (%)	2 (4)	1 (3)
Ever smoking, n (%)	40 (80)	14 (45)
Systolic blood pressure at T0, (mm Hg), mean (SD)	122 (14)	118 (11)
Systolic blood pressure at T11, (mm Hg), mean (SD)	130 (14)	124 (11)
Cholesterol at T0 (mmol/L), mean (SD)	5.5 (0.9)	5.4 (1.1)
Cholesterol at T11 (mmol/L), mean (SD)	5.5 (1.1)	5.5 (1.2)
Statin treatment at T0, n (%)	1 (2)	0
Statin treatment at T11, n (%)	9/45 (20)	7 (23)
BMI at T11 (kg/m^2), mean (SD)	25.6 (4.6)	26.9 (4.9)
IMT at T0 (mm), mean (SD)	0.51 (0.012)	0.55 (0.012)
IMT at T11 (mm), mean (SD)	0.69 (0.016)	0.63 (0.013)
RA variables		
ESR T0 (mm/h), median (Q1-Q3)	11 (8-26)	NA
ESR T11 (mm/h), median (Q1-Q3)	12 (10-22)	NA
CRP T0 (mg/L), median (Q1-Q3)	10 (8.5-14)	NA
CRP T11 (mg/L), median (Q1-Q3)	3.4 (0.9-7.5)	NA
Tender joint count T0 (n), median (Q1-Q3)	2 (0-6)	NA
Tender joint count T11 (n), median (Q1-Q3)	0 (0-2)	NA
Swollen joint count T0 (n), median (Q1-Q3)	3 (1-6)	NA
Swollen joint count T11 (n), median (Q1-Q3)	0 (0-2)	NA
DAS28 T0, median (Q1-Q3)	3.5 (2.6-4.5)	NA
DAS28 T11, median (Q1-Q3)	2.6 (1.8-3.7)	NA
HAQ T0, median (Q1-Q3)	0.5 (0.13-0.88)	NA
HAQ T11, median (Q1-Q3)	0.13 (0-0.63)	NA
PTPN22 1858 T allele carrier, yes (%)	22/49 (45)	NA
Shared epitope, (n) (%)	28/49 (57)	NA
Positive anti-CCP, n (%)	32 (64)	NA

RA: rheumatoid arthritis; SD: standard deviation; T0: baseline; T11: eleven years from baseline; BMI: body mass index; IMT: intima-media thickness; ESR: erythrocyte sedimentation rate; Q1: first quartile; Q3: third quartile; NA: not applicable; CRP: C-reactive protein; DAS28: disease activity score of 28 joints; HAQ: health assessment questionnaire; Anti-CCP: anti-CCP2 antibodies.

Table II. Univariable regression models in 50 patients with RA. $CD4^+CD28null$ was analysed in univariable logistic regression models with $CD4^+CD28null$ >2% as dependent variable. $CD8^+CD28null$ was analysed in univariable linear regression models with the percentage of $CD8^+CD28null$ as dependent variable.

VARIABLE	$CD4^+$ CD28null ≥2%, yes (logistic) Exp. B (CI 95%)	$CD8^+$ % (linear) B (CI 95%)
Age, year	**1.1** (1.0;1.2)**	**1.2** (0,63;1.8)***
Positive CMV IgG at T0, yes	**3.8** (6.0;189)***	**25** (13;36)***
Positive anti-CCP, yes	**0.25** (0.07;0.94)*	-9.6 (-23;3.3)
Shared epitope, yes	2.0 (0.63;6.3)	-4.4 (-17;8.4)
PTPN22, 1858 T allele carrier, yes	1.6 (0.51;5.1)	2.6 (-10;16)
Ever smoking, yes	1.5 (0.37;6.0)	6.2 (-9.5;22
DAS28 at T11	0.99 (0.62;1.6)	3.3 (-2.1;8.9)
Erosive disease at onset, yes	0.54 (0.14;2.0)	-7.7 (-23;8.2)
BMI, kg/m^2	0.97 (0.85;1.1)	-0.45 (-1.4;2.5)

Values of B for $CD4^+CD28null$ and $CD8^+CD28null$ T-cells are not comparable, due to logistic regression and linear regression, respectively.
CI: confidence interval; RA: rheumatoid arthritis; T0: baseline; T11: follow-up eleven years from baseline; CMV: cytomegalovirus; DAS28: disease activity score of 28 joints; BMI: body mass index.
*p<0.05. **p<0.01. ***p<0.001.

CX3CR1 AF647 (2A9-1, BioLegend), together with CD28 PE (CD28.2, BD), or alternatively isotype control PE (MOPC-21, BD), on ice for 20 minutes, followed by washing with FACS-buffer. Following the staining procedure, samples were transferred to 5ml polystyrene tubes and analysed on a LSRII flow cytometer (Becton-Dickinson, San Jose, CA). Obtained data was analysed using the BDDiva software.

CMV-analysis: enzyme-linked immunosorbent assay

Analysis of anti-CMV antibodies of IgG subtypes was done from frozen plasma samples collected at T0 and T11, stored at -80°C. An in-house enzyme linked immune-assay was used as previously described (29, 30). Optical density values below 0.2 were considered negative, based on extensive analysis of sera from CMV-positive and CMV-negative blood donors, where mean optical density plus two standard deviations never exceeded 0.2 in seronegative reference sera. Paired samples from patients and matched controls were assessed on the same plate. For quality control, a standard curve was run on each plate.

Statistics

Descriptive data is presented as median or mean, depending on distribution. For analysis of the association between $CD4^+CD28null$ T-cells and other variables, the patients were classified as having normal (<2%) or elevated (≥2%) percentage of CD28null cells in the $CD3^+CD4^+$ population. The classification was based on previous studies of $CD4^+CD28null$ T-cells, where the median percentage was 1.7–1.9% in healthy normal population and in healthy elderly individuals (31, 32). Linear and logistic regression models were used for analysis of associations between variables. Goodness of fit of multivariable logistic regression models was tested with Hosmer-Lemeshow test. In multivariable regression models, traditional cardiovascular risk factors were included at first, but variables that lowered R^2 were excluded from the models. For comparison of mean or median values between groups, Mann-Whitney U-test or students's t-test was used, depending on distribution. Kruskal-Wallis test was used when more than two groups were compared, Bonferroni corrected in pairwise comparisons. SPSS v. 21 and v. 25 (IBM SPSS INC, Chicago, Illinois, USA) was used for calculations.

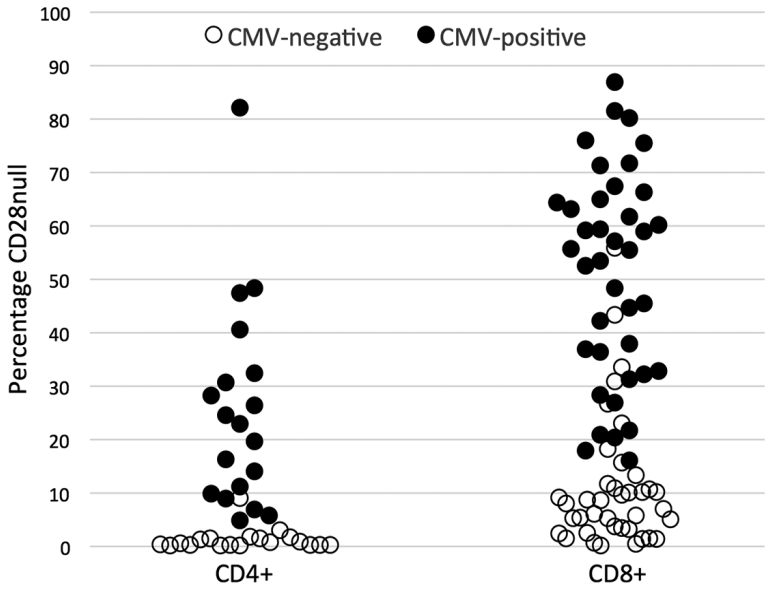

Fig. 1. Percentage of CD28null cells in $CD4^+$ and $CD8^+$ T-cells. Flow-cytometry analysis was performed in peripheral blood from 50 patients with RA eleven years after diagnosis.

Table III. Multivariable regression models in 50 patients with RA eleven years after onset. $CD4^+CD28null$ is analysed in a logistic regression model with $CD4^+CD28null$>2% as dependent variable. $CD8^+CD28null$ is analysed in a linear regression model with the percentage of $CD8^+CD28null$ as dependent variable.

	$CD4^+CD28null$ >2%, yes (logistic)	$CD8^+CD28null$, % (linear)
Variable	Exp. B (CI 95%)	B (CI 95%)
Age, year	1.0 (0,95; 1.15)	**0.75** (0.1; 1.4)*
Anti-CCP, yes	0.41 (0.07; 2.3)	-2.1 (-13.3; 9.2)
Positive CMV IgG at T0, yes	**20.3** (3.2; 130)**	**16.2** (3.1; 29.3)*

Values of B for $CD4^+CD28null$ and $CD8^+CD28null$ T-cells are not comparable, due to different statistical methods (linear *vs*. logistic regression).
Exp. B: exponentiation of the B coefficient; CI: confidence interval; Anti-CCP: anti-CCP2 antibodies; CMV: cytomegalovirus; T0: baseline. *p<0.05. **p<0.01.
Nagelkerke R^2=0.55 for logistic regression model with CD4+CD28null as dependent variable. Hosmer-Lemeshow p-test=0.86 for the model. Adjusted R^2=0.32 for linear regression model with CD8+CD28null as dependent variable.

Table IV. Univariable regression models in 50 patients with RA, with IMT at T11 (1/10 mm) and change in IMT between T0 and T11 (1/10 mm) as dependent variables.

	IMT at T11	Change in IMT from T0 to T11
Variable	B (CI 95%)	B (CI 95%)
Age, year	**0.1** (0.07; 0.1)***	**0.04** (0.008; 0.07)*
Systolic blood pressure T0, mmHg	**0.04** (0.01; 0.06)**	0.01 (-0.009; 0.03)
Systolic blood pressure T11, mmHg	**0.05** (0.02; 0.08)**	0.0002 (-0.02; 0.02)
$CD4^+CD28null$ T-cells >2%, yes	**0.9** (0.05; 1.8)*	0.2 (-0.4; 0.8)
$CD8^+CD28null$ T-cells, %	**0.02** (0.004; 0.04)*	**0.02** (0.004; 0.03)*
$CX3CR1^+$ of $CD4^+$, %	0.02 (-0.03; 0.07)	0.004 (-0.03; 0.04)
$CX3CR1^+$ of $CD8^+$, %	0.02 (-0.001; 0.04)	**0.02** (0.001; 0.03)*
Positive CMV IgG at T0, yes	**1.0** (0.1; 2.0)*	0.4 (-0.3; 1.0)

CI: confidence interval; T0: baseline; T11: follow-up 11 years from baseline; CMV: cytomegalovirus; IMT: intima-media thickness. *p<0.05. **p<0.01. ***p<0.001.

Results

CD28null T-cell frequencies

The median percentage of $CD4^{+-}$CD 28null T-cells was 3.7% (range 0.2–46) and 29 patients (58%) had more than 2% $CD4^{+}$CD28null (Fig. 1). The mean percentage of CD8+CD28null T-cells was 43% (range 4.9–87) (Fig. 1). Table II presents univariable linear regression models exploring factors associated with numbers of CD28null T-cells. In multivariable regression models, CMV-IgG positivity was strongly associated with an increased percentage of CD28null cells in both $CD4^{+}$ and $CD8^{+}$ T-cells (Table III).

IMT and CD28null T-cells in univariable models

IMT at T11 was 0.63 mm in patients with less than 2% CD28null of the $CD4^{+}$ T-cells, *versus* 0.72 mm in patients with $CD4^{+}$CD28null T-cells ≥2% ($p<0.05$). In univariable linear regression models, IMT at T11 was significantly associated with CMV-positivity and increased CD28null T-cells, both $CD4^{+}$ and $CD8^{+}$ (Table IV). IMT at T11 was also significantly associated with age and systolic blood pressure at T0 and T11. No other traditional cardiovascular risk factors (Table I) were significantly associated with IMT (data not shown).

The mean increase in IMT from baseline to T11 (delta IMT) was 0.15 mm and 0.18 mm in patients with <2% and ≥2% $CD4^{+}$CD28null T-cells, respectively ($p=0.42$). Delta IMT was significantly associated with the percentage of $CD8^{+}$CD28null and $CD8^{+}CX3CR1^{+}$ T-cells in univariable linear regression models (Table IV). Delta IMT was associated with age, but not with any other traditional cardiovascular risk factor (data not shown).

IMT and CD28null in multivariable models

Both $CD4^{+}$CD28null ≥2% and the percentage of CD8+CD28null were associated with IMT in multivariable linear regression models that also included systolic blood pressure at T11 (Table V, model 1 and 3). When age was included in the models, no other variable was significantly associated with IMT at T11 (Table V, models 2, 4, 6 and 8). Inclusion of other traditional risk factors but age and systolic blood pressure did not improve the models.

IMT in relation to $CD4^{+}$CD28null T-cells and anti-CCP

To study a potential interaction between anti-CCP-positive RA and $CD4^{+}$CD28null T-cells, patients were categorised according to presence of anti-CCP and CD28null <2% or ≥2%. There were no significant differences in median IMT at T11 between the groups (data not shown).

CMV: associations with T-cell phenotypes

At T0, 33 patients (66%) and 19 controls (66%) had detectable IgG antibodies against CMV. Antibody levels did not differ between patients and controls. CMV-positivity was highly associated with increased prevalence of CD28null T-cells in patients with RA (Table II and III), but also to expression of CX3CR1 in both $CD4^{+}$ and $CD8^{+}$ T-cells (data not shown). When CX3CR1 was analysed in $CD4^{+}$ and $CD8^{+}$ T-cells categorised by CD28-status, only $CD8^{+}$CD28null$CX3CR1^{+}$ T-cells were associated with CMV (data not shown). CMV-positivity was in patients significantly associated with age (52 years *vs*. 41 for CMV-negative patients), but otherwise not associated with any other traditional cardiovascular risk factor or with inflammation in patients or controls.

CX3CR1 in relation to CD28null and IMT

The proportion of cells expressing CX-3CR1 was 75% in $CD4^{+}$CD28null T-cells and 2.4% in $CD4^{+}CD28^{+}$ T-cells respectively, whereas the numbers were 73% and 8.4% in $CD8^{+}$CD28null and $CD8^{+}CD28^{+}$ T-cells respectively. Expression of CX3CR1 in $CD4^{+}$ T-cells, regardless of CD28 status, was not associated with IMT or delta IMT (Table IV). The percentage of $CD8^{+}$ T-cells

Table V. Multivariable linear regression models in 50 patients with RA, with IMT at T11 (1/10 mm) as dependent variable.

	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7	Model 8
Variable	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)
$CD4^{+}$CD28null≥2%, yes	**0.93** (0.12; 1.7)*	0.1 (-0.7; 0.9)						
$CD8^{+}$CD28null, %			**0.02** (0.006; 0.04)**	0.003 (-0.02; 0.02)				
Positive CMV IgG at T0, yes					**1.2** (0.4;2.0)***	0.2 (-0.8; 1.1)		
$CD8^{+}$CX3CR1, %							**0.02** ($3x10^{-5}$; 0.04)*	-0.0007 (-0.02; 0.02)
Systolic BP T11, mmHg	**0.05** (0.02; 0.07)**	0.02 (-0.07; 0.05)	**0.05** (0.02; 0.07)**	0.02 (-0.007; 0.05)	**0.05** (0.02.; 0.08)***	0.02 (-0.01; 0.05)	**0.05** (0.02; 0.07)**	0.02 (-0.008; 0.05)
Age, year		**0.09** (0.05; 0.1)***		**0.09** (0.04; 0,1)***		**0.09** (0.04; 0.14)***		**0.1** (0.05; 0.14)***

CI: confidence interval; CMV: cytomegalovirus; BP: blood pressure; T0: baseline; T11: follow-up 11 years from baseline.
Adjusted R^2 model 1=0.23. Adjusted R^2 model 2=0.42. Adjusted R^2 model 3=0.25. Adjusted R^2 model 4=0.42. Adjusted R^2 model 5=0.28. Adjusted R^2 model 6=0.42. Adjusted R^2 model 7=0.21. Adjusted R^2 model 8=0.42. *$p<0.05$. **$p<0.01$. ***$p<0.001$.

expressing CX3CR1 ($CD8^+CX3CR1^+$) was significantly associated with IMT in a multivariable linear regression model (Table V, model 7), but when adjusted for age, neither $CD8^+CX3CR1^+$ T-cells nor systolic blood pressure at T11 were statistically significant (Table V, model 8). $CD8^+CX3CR1^+$ T-cells were also significantly associated with delta IMT (Table IV), but not adjusted for age (data not shown). When expression of CX3CR1 was analysed in $CD8^+CD28$null and $CD8^+CD28^+$ T-cells separately, it was not associated with IMT or delta IMT (data not shown). Inflammatory variables (ESR, CRP, swollen joint count, tender joint count, DAS28 at T0 and T11) were not associated with expression of CX3CR1, regardless of $CD4^+$, $CD8^+$ or CD28-status, (data not shown).

CMV, IMT and increase in IMT

In linear regression models, CMV-IgG positivity was associated with IMT, although not when adjusted for age (Table V, models 5 and 6). In RA-patients with $CD4^+CD28$null <2%, IMT was not associated with CMV-status (data not shown). When patients and controls were grouped according to CMV-IgG status, the median values of IMT at T0, T1.5 and T11 were as presented in Figure 2. CMV-IgG positive patients had a rapid increase in IMT during the first 1.5 years of RA, statistically significantly different from controls (both CMV-IgG negative and CMV-IgG positive) and close to statistically significant different (p=0.075) from CMV-negative patients. There were no statistically significant differences between the groups in terms of age, sex, diabetes, blood pressure, blood lipids or diabetes.

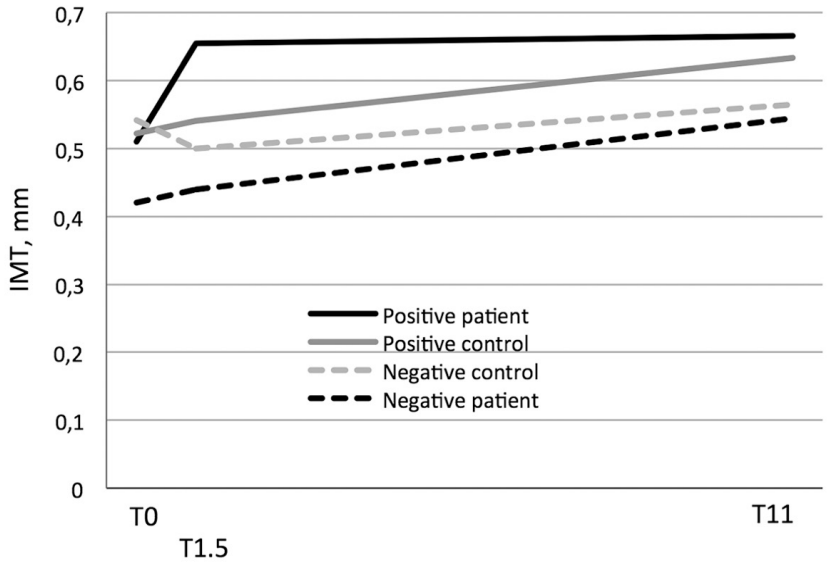

Fig. 2. Median IMT during 11 years of follow-up. Results from carotid ultrasound in 20 patients with RA and 17 controls, grouped by CMV-status. Pairwise comparisons of change in IMT from T0 to T1.5 (CMV-positive patients as reference): CMV-positive controls p=0.06; CMV-negative controls p=0.01; CMV-negative patients p=0.08. CMV-positive patients n=10; CMV-positive controls n=12; CMV-negative controls n=5; CMV-negative patients n=10.

Discussion

Atherosclerosis is an inflammatory process, where T-cells are involved (7). In this study of subclinical atherosclerosis in patients with RA, an elevated percentage of T-cells lacking the co-stimulatory molecule CD28 (CD28null T-cells) was associated with increased IMT. CD28null T-cells differ from normal $CD28^+$ in several aspects; $CD4^+CD28$null T-cells have cytotoxic properties, whereas $CD8^+CD28$null T-cells can exhibit both increased and decreased cytotoxicity (33). Previous studies of atherosclerosis and CD28null T-cells have focused on $CD4^+CD28$null T-cells, but we assessed both $CD4^+CD28$null and $CD8^+CD28$null T-cells, and found increased frequency of either of them to be associated with a higher IMT. In previous studies in patients with RA, atherosclerosis and $CD4^+CD28$null T-cells were associated (12, 13, 34). $CD8^+CD28$null T-cells were assessed in one of these studies (13) and found to be associated with atherosclerosis, although not significantly associated when adjusted for traditional cardiovascular risk factors. In a study in the general population, increased numbers of $CD8^+CD28$null T-cells were associated with coronary artery disease (15). The ratio of $CD4^+$/$CD8^+$ T-cells is 1/1 in advanced atherosclerotic lesions, compared to 2/1 in peripheral blood (14), indicating that $CD8^+$T-cells are involved in the process of atherosclerosis.

In line with previous studies, an elevated percentage of CD28null T-cells was associated with CMV positivity (17-21). It has been hypothesised that CMV infection, via T-cell abnormalities, is the cause of increased atherosclerosis in patients with RA (18). Our results to some extent support this hypothesis, as there are significant associations between CD28null T-cells and IMT, between CMV-IgG positivity and CD28null T-cells, and between CMV-IgG positivity and IMT. Yet we cannot say whether the proposed mechanism is correct. Of note, CD28null T-cells represent a useful 'proxy' for a cell differentiation state, but still selects a heterogenous T cell population, which probably should be better defined in the future.

Our observations imply that the impact of CMV-infection on atherosclerosis in patients with RA and in the general population is different. As shown in Figure 2, CMV-IgG positive RA-patients had a rapid increase in IMT after onset of RA, whereas the CMV-positive and CMV-negative controls did not differ in progress of IMT. In studies of the general population, CMV-positivity in combination with an inflammatory burden, was associated with an increased risk of coronary artery disease and death thereof, whereas CMV-IgG positivity alone, without an increased inflammation, was not associated with such events (35, 36).

A possible explanation to the observation of a rapid increase in IMT in

CMV-IgG positive patients after onset of RA, is an interaction on endothelial activation between RA and CMV-infection. Inflammation in RA is known to cause endothelial activation, that promotes inflammatory cell adhesion and migration, and thus promote atherosclerosis (6). CMV infects endothelial cells and causes their rapid activation and increased expression of adhesion molecules, although the activation following CMV-infection is not constant, but later is down-regulated (37). When a CMV-infected individual develops RA, the systemic inflammation activates endothelial cells that are already CMV-infected. The inflammatory load might cause an up-regulation of the endothelial activation from CMV, thus causing a high grade of activation and a subsequent rapid increase in IMT. Reactivation of latent CMV due to altered immune system and inflammation, could be another explanation to a rapid increase in IMT in CMV-positive patients with new-onset RA. This needs to be further explored in studies of endothelial cell function and activation in patients with RA, with known status of CMV-infection.

The rapid increase in IMT seen after onset of RA in CMV-IgG positive patients, suggests that interventional studies with antiviral medication should be considered. One such study has been initiated in patients with vasculitis (38), but to the best of our knowledge, no such study in patients with RA is performed or planned, although it could provide an opportunity to treat the increased risk of atherosclerosis in patients with RA.

Expression of CX3CR1 was associated with CMV-IgG positivity in our study as in previous studies of the general population (39). In both $CD4^+$ and $CD8^+$ T-cells, CX3CR1 was detectable in a majority of CD28null T-cells, whereas the expression of CX3CR1 in $CD28^+$ T-cells was low. However, CX3CR1 was not significantly associated with IMT in groups of T-cells stratified by CD28-status, at variance with a previous study in patients with RA (23). This is a bit surprising, since the fractalkine-CX3CR1 interaction promotes T-cell-adhesion to activated endothelium, thus facilitating inflammation in the arterial wall (25). Our results do not confirm that expression of CX3CR1 is more than a marker of CD28null T-cells in terms of IMT. Neither do our results support the association between disease activity and CX3CR1 status that was found in the previous study in patients with RA (23). Of note, different expression levels of CX3CR1 have recently been suggested to distinguish unique memory subsets in the $CD8^+$ T cell population in mice (40) and humans (41) and this could tentatively explain the different results.

Presence of anti-CCP was not associated with an elevated percentage of CD28null T-cells in our study, nor in previous studies (42, 43). Neither did patients carrying the shared epitope HLA or the PTPN22 1858 T-allele have increased levels of CD28null T-cells in our study. The relation between shared epitope and $CD4^+$CD28null T-cells has been investigated previously, with conflicting results (10, 44). Since both shared epitope and the PTPN22 1858T allele are risk factors for anti-CCP-positive RA, it is reasonable to think of anti-CCP-positivity and CD28null T-cells as independent immunological alterations, although a recent study showed that the PTPN22 1858T allele was associated with cytotoxic features in both $CD4^+$ and $CD8^+$ T-cells (45). In our cohort, no interaction on IMT between anti-CCP antibodies and $CD4^+$CD28null T-cells was seen, although this has been proposed by others (43).

In the present study, traditional risk factors but age had little impact on IMT. However, this could in part be explained by the fact that primary preventive measures are undertaken, making the levels of blood lipids and blood pressure lower. The number of patients treated with statins increased remarkably from T0 to T11, but the average levels of blood lipids was equal. Since treatment with statins probably has been initiated in individuals with a high cardiovascular risk, and are efficient in lowering the levels of blood cholesterol, low levels of blood cholesterol in statin treated patients are a marker of a high risk, thus confounding the analysis.

In our study, presence of plaques was not assessed. This is a limitation, since this measure predicts atherosclerotic cardiovascular events in patients with RA (46) and also identifies patients with a higher estimated cardiovascular risk (47). The latter association has also been shown in patients with ankylosing spondylitis (48, 49). Another limitation is the relatively small number of participants. In particular, the number of controls is small, but the focus of the ongoing longitudinal study is to analyse factors associated with atherosclerosis in patients with RA, not in the general population. This also explains why analysis of T-cell populations was done in patients, but not in controls. One strength of the study is the prospective design. Most studies of atherosclerosis in RA have cross-sectional design, but our prospective study gives a unique possibility to analyse change over time. Furthermore, this cohort of patients with RA is unselected and population-based: all patients with new-onset RA, aged 60 years old or less, in the northernmost region of Sweden were invited to participate in the study when it was initiated. Another strength is that every ultrasound examination has been performed by the same experienced examinator.

In conclusion, CMV-IgG positivity in patients with RA is associated with an increased frequency of $CD4^+$ and $CD8^+$ CD28null T-cells and with an increased atherosclerosis measured by IMT. Our study suggests that CMV-positive patients with RA comprise a high-risk population in terms of cardiovascular disease. CMV serological status and frequency of CD28null T-cells should be considered in future studies of atherosclerosis and cardiovascular disease in patients with RA, and the possibility of a protective effect of antiviral treatment should be considered.

Acknowledgements

The authors thank Monika Hansson, Department of Medicine, Rheumatology Unit, Center for Molecular Medicine, Karolinska Institutet for laboratory analyses, Elisabet Lundström, Department of Surgical and Perioperative Sciences/Clinical physiology for ultrasound examinations and Professor

Solbritt Rantapää-Dahlqvist, Department of Public Health and Clinical Medicine/Rheumatology, Umeå University, for genetic data on the patients.

References

1. WALLBERG-JONSSON S, OHMAN ML, DAHLQVIST SR: Cardiovascular morbidity and mortality in patients with seropositive rheumatoid arthritis in Northern Sweden. *J Rheumatol* 1997; 24: 445-51.
2. AVINA-ZUBIETA JA, THOMAS J, SADATSAFAVI M, LEHMAN AJ, LACAILLE D: Risk of incident cardiovascular events in patients with rheumatoid arthritis: a meta-analysis of observational studies. *Ann Rheum Dis* 2012; 71: 1524-9.
3. AVIÑA-ZUBIETA JA, CHOI HK, SADATSAFAVI M, ETMINAN M, ESDAILE JM, LACAILLE D: Risk of cardiovascular mortality in patients with rheumatoid arthritis: A meta-analysis of observational studies. *Arthritis Care Res* 2008; 59: 1690-97.
4. WÅLLBERG-JONSSON S, BACKMAN C, JOHNSON O *et al*.: Increased prevalence of atherosclerosis in patients with medium term rheumatoid arthritis. *J Rheumatol* 2001; 28: 2597-602.
5. VAN SIJL AM, PETERS MJ, KNOL DK *et al*.: Carotid intima media thickness in rheumatoid arthritis as compared to control subjects: a meta-analysis. *Semin Arthritis Rheum* 2011; 40: 389-97.
6. SKEOCH S, BRUCE IN: Atherosclerosis in rheumatoid arthritis: is it all about inflammation? *Nat Rev Rheumatol* 2015; 11: 390-400.
7. HANSSON GK, HERMANSSON A: The immune system in atherosclerosis. *Nat Immunol* 2011; 12: 204-12.
8. FONSEKA CY, RAO DA, TESLOVICH NC *et al*.: Mixed-effects association of single cells identifies an expanded effector $CD4^+$ T cell subset in rheumatoid arthritis. *Sci Transl Med* 2018; 10: eaaq0305.
9. ISHIGAKI K, SHODA H, KOCHI Y *et al*.: Quantitative and qualitative characterization of expanded $CD4^+$ T cell clones in rheumatoid arthritis patients. *Sci Rep* 2015; 5: 12937.
10. FASTH AER, CAO D, VAN VOLLENHOVEN R, TROLLMO C, MALMSTRÖM V: CD28null$CD4^+$ T cells – Characterization of an effector memory T-cell population in patients with rheumatoid arthritis. *Scand J Immunol* 2004; 60: 199-208.
11. DUMITRIU IE, ARAGUÁS ET, BABOONIAN C, KASKI JC: $CD4^+$ CD28 null T cells in coronary artery disease: when helpers become killers. *Cardiovasc Res* 2009; 81: 11-9.
12. GERLI R, SCHILLACI G, GIORDANO A *et al*.: $CD4^+CD28^-$ T lymphocytes contribute to early atherosclerotic damage in rheumatoid arthritis patients. *Circulation* 2004; 109: 2744-8.
13. WINCHESTER R, GILES JT, NATIV S *et al*.: Association of elevations of specific T cell and monocyte subpopulations in rheumatoid arthritis with subclinical coronary artery atherosclerosis. *Arthritis Rheumatol* (Hoboken NJ) 2016; 68: 92-102.
14. GEWALTIG J, KUMMER M, KOELLA C, CATHOMAS G, BIEDERMANN BC: Requirements for CD8 T-cell migration into the human arterial wall. *Hum Pathol* 2008; 39: 1756-62.
15. JONASSON L, TOMPA A, WIKBY A: Expansion of peripheral $CD8^+$ T cells in patients with coronary artery disease: relation to cytomegalovirus infection. *J Intern Med* 2003; 254: 472-8.
16. YU HT, YOUN J-C, KIM JH *et al*.: Arterial stiffness is associated with cytomegalovirus-specific senescent $CD8^+$ T cells. *J Am Heart Assoc* 2017; 6.
17. HOOPER M, KALLAS EG, COFFIN D, CAMPBELL D, EVANS TG, LOONEY RJ: Cytomegalovirus seropositivity is associated with the expansion of $CD4^+CD28$- and $CD8^+CD28^-$ T cells in rheumatoid arthritis. *J Rheumatol* 1999; 26: 1452-7.
18. BROADLEY I, PERA A, MORROW G, DAVIES KA, KERN F: Expansions of cytotoxic $CD4^+CD28^-$ T cells drive excess cardiovascular mortality in rheumatoid arthritis and Other chronic inflammatory conditions and are triggered by CMV infection. *Front Immunol* 2017; 8: 195.
19. GRATAMA JW, NAIPAL AM, OOSTERVEER MA *et al*.: Effects of herpes virus carrier status on peripheral T lymphocyte subsets. *Blood* 1987; 70: 516-23.
20. DERHOVANESSIAN E, MAIER AB, HÄHNEL K *et al*.: Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated $CD4^+$ and $CD8^+$ T-cells in humans. J *Gen Virol* 2011; 92: 2746-56.
21. KOCH S, LARBI A, ÖZCELIK D *et al*.: Cytomegalovirus infection. *Ann NY Acad Sci* 2007; 1114: 23-35.
22. DAHAL U, SHARMA D, DAHAL K: An unsettled debate about the potential role of infection in pathogenesis of atherosclerosis. *J Clin Med Res* 2017; 9: 547-54.
23. PINGIOTTI E, CIPRIANI P, MARRELLI A *et al*.: Surface expression of Fractalkine Receptor (CX3CR1) on $CD4^+/CD28^-$ T cells in RA patients and correlation with atherosclerotic damage. *Ann NY Acad Sci* 2007; 1107: 32-41.
24. FONG AM, ROBINSON LA, STEEBER DA *et al*.: Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med* 1998; 188: 1413-9.
25. VAN DE BERG PJEJ, YONG S-L, REMMERSWAAL EBM, VAN LIER RAW, TEN BERGE IJM: Cytomegalovirus-Induced effector T cells cause endothelial cell damage. *Clin Vaccine Immunol* 2012; 19: 772-9.
26. SÖDERGREN A, KARP K, BOMAN K *et al*.: Atherosclerosis in early rheumatoid arthritis: very early endothelial activation and rapid progression of intima media thickness. *Arthritis Res Ther* 2010; 12: R158.
27. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The american rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
28. BOMAN A, KOKKONEN H, ÄRLESTIG L, BERGLIN E, RANTAPÄÄ-DAHLQVIST S: Receptor activator of nuclear factor kappa-B ligand (RANKL) but not sclerostin or gene polymorphisms is related to joint destruction in early rheumatoid arthritis. *Clin Rheumatol* 2017; 36: 1005-12.
29. SUNDQVIST VA, WAHREN B: Development of sensitive and enzyme-linked immunoassays for herpesvirus antigens and some applications. *Dev Biol Stand* 1982; 52: 245-53.
30. RAHBAR AR, SUNDQVIST V-A S, WIRGART BZ, GRILLNER L, SÖDERBERG-NAUCLER C: Recognition of cytomegalovirus clinical isolate antigens by sera from cytomegalovirus-negative blood donors. *Transfusion* (Paris) 2004; 44: 1059-66.
31. SCHMIDT D, GORONZY JJ, WEYAND CM: $CD4^+$ $CD7^-$ $CD28^-$ T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* 1996; 97: 2027-37.
32. GORONZY JJ, FULBRIGHT JW, CROWSON CS, POLAND GA, O'FALLON WM, WEYAND CM: Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals. *J Virol* 2001; 75: 12182-7.
33. MOU D, ESPINOSA J, LO DJ, KIRK AD: CD28 Negative T cells: is their loss our gain? *Am J Transplant* 2014; 14: 2460-6.
34. WARRINGTON KJ, KENT PD, FRYE RL *et al*.: Rheumatoid arthritis is an independent risk factor for multi-vessel coronary artery disease: a case control study. *Arthritis Res Ther* 2005; 7: R984-91.
35. BLANKENBERG S, RUPPRECHT HJ, BICKEL C *et al*.: Cytomegalovirus infection with interleukin-6 response predicts cardiac mortality in patients with coronary artery disease. *Circulation* 2001; 103: 2915-21.
36. ZHU J, QUYYUMI AA, NORMAN JE, CSAKO G, EPSTEIN SE: Cytomegalovirus in the pathogenesis of atherosclerosis: The role of inflammation as reflected by elevated C-reactive protein levels. *J Am Coll Cardiol* 1999; 34: 1738-43.
37. JEFFERY HC, SÖDERBERG-NAUCLER C, BUTLER LM: Human cytomegalovirus induces a biphasic inflammatory response in primary endothelial cells. *J Virol* 2013; 87: 6530-5.
38. CHANOUZAS D, DYALL L, NIGHTINGALE P *et al*.: Valaciclovir to prevent Cytomegalovirus mediated adverse modulation of the immune system in ANCA-associated vasculitis (CANVAS): study protocol for a randomised controlled trial. *Trials* 2016; 17: 338.
39. VIEIRA BRAGA FA, HERTOGHS KM, VAN LIER RAW, VAN GISBERGEN KP: Molecular characterization of HCMV-specific immune responses: Parallels between $CD8^+$ T cells, $CD4^+$ T cells, and NK cells. *Eur J Immunol* 2015; 45: 2433-45.
40. GERLACH C, MOSEMAN EA, LOUGHHEAD SM *et al*.: The chemokine receptor CX3CR1 defines three antigen-experienced CD8 T cell subsets with distinct roles in immune surveillance and homeostasis. *Immunity* 2016; 45: 1270-84.
41. CONROY MJ, MAHER SG, MELO AM *et al*.: Identifying a novel role for fractalkine (CX-3CL1) in memory $CD8^+$ T cell accumulation in the omentum of obesity-associated cancer patients. *Front Immunol* 2018; 9: 1867.
42. FASTH AE, SNIR O, JOHANSSON AA *et al*.: Skewed distribution of proinflammatory $CD4^+CD28$null T cells in rheumatoid arthri-

tis. *Arthritis Res Ther* 2007; 9: R87.
43. GERLI R, VAUDO G, BOCCI EB, SCHILLACI G, BISTONI O, SHOENFELD Y: Different roles for anti–cyclic citrullinated peptide antibodies and CD4+CD28null cells in the acceleration of atherosclerosis in rheumatoid arthritis: Comment on the article by Farragher *et al*. *Arthritis Rheum* 2009; 60: 631-2.
44. CHAPMAN A, STEWART SJ, NEPOM GT *et al*.: $CD11b^+CD28^-CD4^+$ human T cells: activation requirements and association with HLA-DR alleles. *J Immunol* 1996; 157: 4771-80.
45. CHEMIN K, RAMSKÖLD D, DIAZ-GALLO L-M *et al*.: EOMES-positive $CD4^+$ T cells are increased in PTPN22 (1858T) risk allele carriers. *Eur J Immunol* 2018; 48: 655-69.
46. EVANS MR, ESCALANTE A, BATTAFARANO DF, FREEMAN GL, O'LEARY DH, DEL RINCON I: Carotid atherosclerosis predicts incident acute coronary syndromes in rheumatoid arthritis. *Arthritis Rheum* 2011; 63: 1211-20.
47. FENT GJ, GREENWOOD JP, PLEIN S, BUCH MH: The role of non-invasive cardiovascular imaging in the assessment of cardiovascular risk in rheumatoid arthritis: where we are and where we need to be. *Ann Rheum Dis* 2017; 76: 1169-75.
48. RUEDA-GOTOR J, LLORCA J, CORRALES A *et al*.: Cardiovascular risk stratification in axial spondyloarthritis: carotid ultrasound is more sensitive than coronary artery calcification score to detect high-cardiovascular risk axial spondyloarthritis patients. *Clin Exp Rheumatol* 2018; 36: 73-80.
49. RUEDA-GOTOR J, QUEVEDO-ABELEDO JC, CORRALES A *et al*.: Reclassification into very-high cardiovascular risk after carotid ultrasound in patients with axial spondyloarthritis. *Clin Exp Rheumatol* 2020; 38: 724-31.