The VICM biomarker is released from activated macrophages and inhibited by anti-GM-CSFRα-mAb treatment in rheumatoid arthritis patients

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Abstract

Objective

Macrophages possess widespread pro-inflammatory, destructive, and remodelling capabilities that can critically contribute to acute and chronic diseases, such as rheumatoid arthritis (RA). Continuous monitoring and measurement of selective counteraction of macrophage activity in patients require a sensitivity and non-invasive marker. We characterised the VICM (citrullinated and MMP degraded vimentin fragment) biomarker by investigating the release from in vitro activated macrophages and by monitoring the change in serum levels after treatment with the anti-GM-CSFRα-mAb (mavrilimumab).

Methods

Peripheral blood mononuclear cells were isolated, and lipopolysaccharide (LPS) was used to activate the macrophages and calcium chloride ($CaCl_2$) was used to facilitate the citrullination process of vimentin. Supernatants, cell lysates, was collected and analysed by ELISA, and western blotting. RA patients were treated with mavrilimumab+methotrexate or methotrexate alone in a phase 2b study (NCT01706926) once every two weeks for 24 weeks. Serum levels of VICM were measured at baseline and multiple time points post-treatment. In addition, whole blood expression of peptidylarginine deiminase-2 (PAD-2) and matrix metalloproteinase-9 (MMP-9) transcripts were tested by quantitative reverse transcriptase PCR assays at day 0 and day 169 post-treatment.

Results

VICM levels were significantly higher at day 5 and 8 in supernatants of activated macrophages compared to controls ($p<0.01$), which was confirmed by Western blot. In RA patients, VICM correlated with disease activity (DAS28), modified total sharp score (mTSS), joint space narrowing (JSN), joint erosions and CRP at baseline. VICM was dose-dependently and significantly ($p<0.01$) inhibited by mavrilimumab. This suppression of VICM serum levels was supported by a decreased expression of PAD2 and MMP9 transcripts in patients treated with mavrilimumab.

Conclusion

These data verified that VICM is released by activated macrophages. Treatment of RA patients with mavrilimumab significantly reduced release of VICM and peptidylarginine deiminases-2 (PAD-2) gene expression indicating that mavrilimumab indeed is targeting activated macrophages and that VICM may be a novel blood-based marker of anti-GM-CSF response.

Key words

macrophages, biomarker, citrullination, vimentin, rheumatoid arthritis, autoimmunity, anti-GM-CSF, matrix metalloproteinase

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic and degenerative inflammation (1, 2). The aetiology is not fully understood. However, a dysregulated immune response with the development of autoantibodies against the cartilage and other extracellular matrix structures is one of the main driving pathogenic factors (1, 3-6). Macrophage involvement has been reported to be a major contributor to the development of RA, with roles in the continuous remodelling and destruction of the periarticular bone and articular cartilage (7, 8). In contrast, during this destructive process of the bone and cartilage in joint, the synovial membrane is affected by chronic inflammation, synovitis, where the constant inflammation and remodelling of the synovial membrane results in increased disposition of collagens and other extracellular matrix proteins (9).

The multiple roles of macrophages in RA joints are partly due to the diverse roles of macrophages within the innate immune system (10). Classically activated macrophages are classified as guardians, which, by inflammatory activation, are constantly on patrol for foreign substances/organisms that are not supposed to be present within the body (10).

Maturation of macrophages is controlled by cytokines, *e.g.* the granulocyte maturation colony stimulating factor (GM-CSF), and macrophage colony stimulating factor (M-CSF) (11, 12). These cytokines will differentiate monocytes into macrophages, after which the microenvironment will determine the fate of the macrophage phenotype (12, 13). For example, ligands that promote classically activated macrophages can be membrane debris from bacteria, *e.g.* the lipopolysaccharide (LPS) (10, 11).

The cytosolic intermediate filament, vimentin, facilitates the movement and structure of the cells. Macrophages also express vimentin. Classically activated macrophages have been shown to have increased expression of the peptidylarginine deiminases (PADs) enzymes that control the conversion of arginine amino acids into citrulline-amino acids in the presence of calcium ions, a process referred to as citrullination (14). Vimentin is highly susceptible for citrullination by classically activated macrophages (14). Moreover, metalloproteinases (MMPs) are also increased in classically activated macrophages, leading to increased degradation of intracellular and extracellular proteins. Recently an MMP-2/8 derived and citrullinated vimentin fragment (VICM) (15), serum biomarker was shown to have potential as a therapeutic efficacy biomarker of RA (16) and ankylosing spondylitis (17).

Mavrilimumab, a human monoclonal antibody targeting the alpha subunit of the GM-CSF receptor (GM-CSFrα), has been evaluated in a phase II randomised, double-blind, placebo-controlled study to investigate efficacy and safety in subjects with rheumatoid arthritis (RA). The phase II study showed that mavrilimumab induced clinically significant responses in RA subjects, suggesting that inhibiting the mononuclear phagocyte pathway may provide a novel therapeutic approach for RA (12, 18, 19). The aim of this study was to gain more insights into the VICM biomarker which was investigated by; 1. characterising of VICM *in vitro*, a blood-based biomarker reflecting activated macrophages, 2. understanding the association between serum VICM levels and clinical assessment scores, and 3. looking at the pharmacodynamic properties of VICM in response to mavrilimumab.

Materials and methods

Serum samples from a RA clinical trial with mavrilimumab

In a parallel-group, double-blind, phase 2b study (NCT01706926; completed), patients with inadequate response to ≥1 traditional disease-modifying antirheumatic drug(s) (DMARDs), Disease Activity Score 28 (DAS28)-C-reactive protein (CRP)/erythrocyte sedimentation rate ≥3.2, ≥4 swollen joints despite treatment with methotrexate (MTX), were randomised 1:1:1:1 to subcutaneous mavrilimumab (150mg, 100mg, or 30mg), or placebo every other week, for 24 weeks (18). Co-primary outcomes were DAS28-CRP change from baseline to week 12, and ACR20 re-

sponse rate (week 24). For the study, 326 patients were randomised (150 mg, n=79; 100 mg, n=85; 30 mg, n=81; placebo (only methotrexate), n=81); 305 patients completed the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonisation Guidance for Good Clinical Practice and approved by appropriate institutional review boards or independent ethics committees at each site. All subjects provided written consent to participate in the study. For a complete description of the methodology and details of this clinical phase IIb study, the reader is advised to consult the publication by Burmester *et al*. and supplementary files (18).

Cell isolation and culture

$CD14^+$ monocytes were isolated from PBMCs from four blood donors with magnetic beads as previously described (20). The isolated $CD14^+$ cells were seeded at a density of 150.000 cells/cm^2 in T175 culture flasks, and differentiated into macrophages for seven days in alpha-MEM culture media with the addition of 10% fetal bovine serum, 1% thymine, 1% penicillin/streptomycin, and 25ng/ml M-CSF. AlamarBlue® was applied, as previously described (21) and in accordance with the supplier, to monitor the viability of the cells during the course of the different stimuli. Culture media was changed every second day. After 5 days of differentiation macrophages (MØ) were seeded in T25 culture flasks, by discarding the culture media followed by 3 times wash in PBS. The cells were passaged by incubating the cells in trypsin for 10–15 mins, and then gently scraped off the plastic and reseeded in T25 culture flasks. To investigate VICM as a potential biomarker for macrophages, LPS was applied for activation of the macrophages and $CaCl_2$ was applied to activate the PAD enzymes that convert the arginine amino acid into a citruline amino acid (VICM is a citrullinated fragment of viementin). The *in vitro* macrophages received either treatments with 100ng LPS (MØ-L), 100ng LPS+$CaCl_2$ (MØ-LC) or 2mM $CaCl_2$ (MØ-C). The MØ-L and MØ-C cultures acted as the controls to control for the effect of mono stimulation of both LPS and $CaCl_2$. To increase accumulation of secreted protein fragments, *e.g*. VICM fragments, the culture media were collected and refreshed at day one, day five and day eight.

Cell lysis and protein extraction

After *in vitro* stimulation, cultured macrophages were trypsinised as previously described. Cells were then transferred to an Eppendorf tube and lysed by adding 50µl RIPA Buffer (0.1% SDS, 50 mM Tris-HCl pH 7.4, 0.25% Sodium deoxycholate, 1 mM EDTA, and 150 mM NaCl) to each tube and incubated for 30 min at 100 motion/min. Each tube was centrifuged at 10,000 RCF at 4°C for 10 minutes and the supernatant was transferred to a new precooled 0.5 mL Eppendorf tube and stored at -20°C until use.

Western blotting

Samples were boiled at 85°C with SDS-PAGE for 10 minutes and spun down. The macrophage lysate samples were loaded on a 10 or 12% SDS gel Novex gel (Life Science). Beta-actin was used as loading control for all samples. The gel was then run at 120 volts for 15 minutes and at 180 V for 45–60 minutes. Samples were blotted with Iblot™ (Invitrogen) onto a nitrocellulose membrane for 7 minutes. Blocking buffer (5% skim milk in TPBS) was added to the blotted nitrocellulose membrane and incubated for 60 min at room temperature on a shaker (50 motion/min). Primary antibody goat-anti-human vimentin (Abcam) was diluted 1:2500 and anti-CD68 (Abcam), anti-MMP-2 (Abcam) anti-MMP-8 (Abcam) was diluted 1:1000 in incubation buffer consisting of 10% blocking buffer and in TPBS and added to the membrane for overnight incubation at 4°C on a rocking shaker. The primary antibody solution was then discarded and the membrane washed three times in TPBS for 5 minutes at 50 motions/min. Secondary antibody was prepared in the same buffer solution as the primary antibody and added to the membrane and incubated for 1 hour at room temperature. The membrane was washed three times for 5 minutes with TPBS at room temperature at 50 motions/min. ECL prime kit (GE Healthcare) was used for protein detection in accordance with the manufacturer's instructions. The membrane was then placed on a transparent sheet and covered with the ECL mixed solution for 5 mins. LI-COR® C-DIGIT was used to expose the proteins.

Competitive enzyme-linked immunosorbent assay

The MMP-2/8 degraded and citrullinated vimentin neo-epitope (VICM) was measured in the serum of RA patients and supernatants from the macrophages cultures by competitive ELISA assay as previously described (17). Briefly, 96 well streptavidin pre-coated microtiter plates (Roche) were coated with the 2.5 ng biotin-labelled VICM neo-epitope peptide (amino acid sequence: biotin-RLRSSVPGV-Citrulline) for 30 min at room temperature at 300 rpm and subsequently washed 5 times in washing buffer (25 mM TRIZMA, 50 mM NaCl, 0.036% Bronidox L5, 0.1% Tween 20, adjusted to pH 7.4 at 20°C) by ELISA washer (BioTek® Instruments, Microplate washer, ELx405 Select CW, Winooski, USA). 20µL of standard peptide (amino acid sequence: RLRSSVPGV-Citrulline), assay controls and unknown samples were diluted accordingly (supernatants from macrophages were run undiluted) in incubation buffer (50mM Tris, 1% BSA, 0.1% Tween-20, 0.36% Bronidox, adjusted to pH 7.4 at 20°C) were then added to the plates together with 100µL of the HRP conjugated primary antibody (monoclonal IgG1 antibody: 4ng/mL) specific for the VICM neo-epitope cleavage site and incubated for 1 hour at room temp. Finally, the plates were washed 5 times in washing buffer and TMB was added and stopped after 15 minutes incubation with a stopping buffer (0.1% SO_2P_4). Since the cell count was different between treatments in the *in vitro* macrophage cultures, VICM supernatant levels were normalised to the cell count of the controls.

Immunofluorescence

Some macrophage cultures were designated for immunofluorescence. When these cultures had reached a confluence

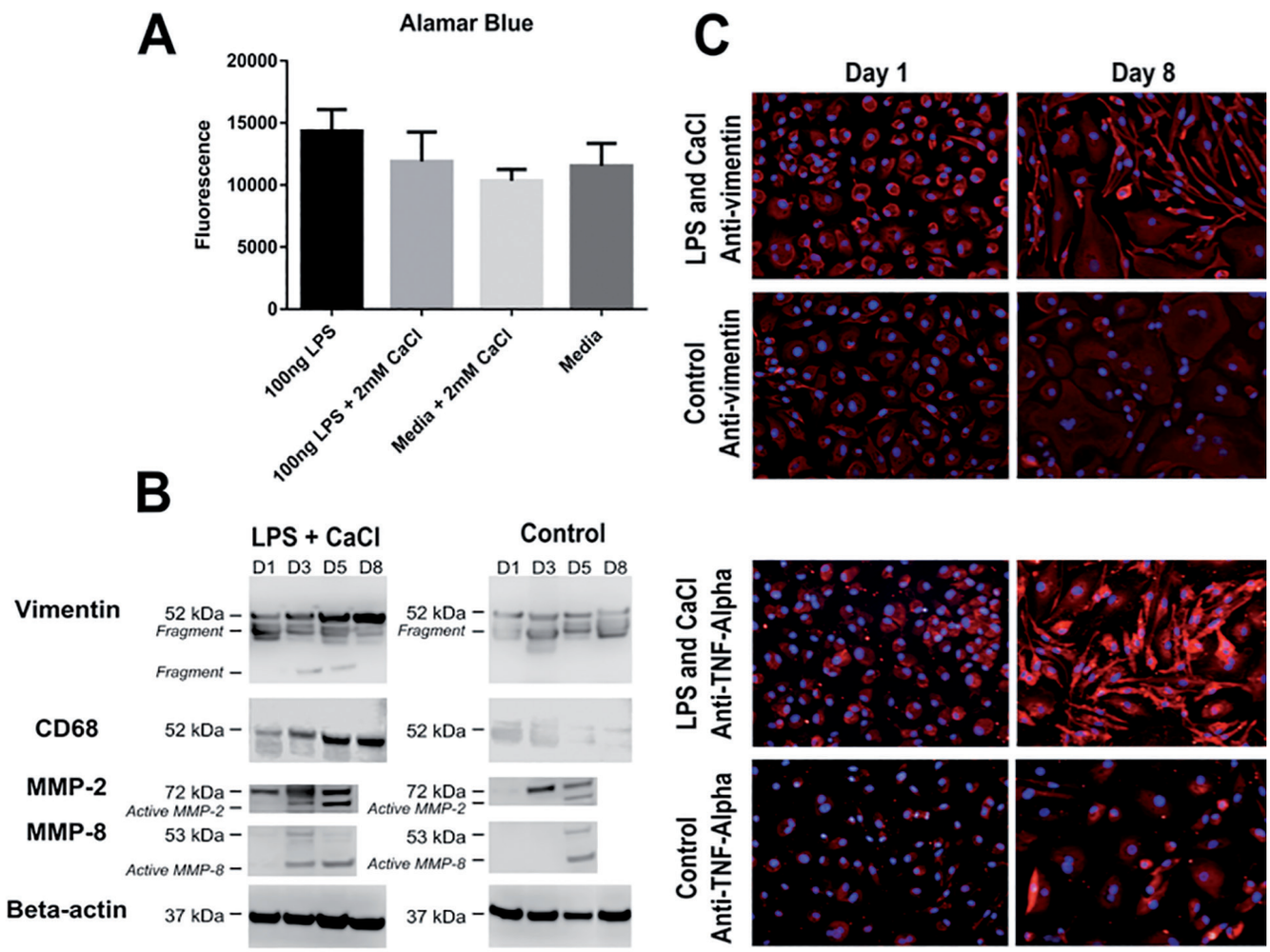

Fig. 1. Viability of the cultured cells *in vitro* with AlamarBlue® assay, and macrophage characterisation by Western blotting and immunofluorescence on different time points. **A**) The viability of the cells was measured in all groups, **B**) protein expression in LPS+$CaCl_2$ treated cells and controls by western blotting, **C**) morphological cell changes anti-vimentin immunofluorescence, anti-TNF-alpha, and DAPI staining on LPS+$CaCl_2$ treated cells and controls. Asterisk (*) indicates different levels of statistical significance; ****p<0.001*. The control western blot for lane 4 (vimentin, CD68, and beta-actin), was run on a separate gel.

level of 70–80% the medium was removed and cells were washed 3 times with 0.3 ml phosphate buffered saline. The cells were fixed with 4% paraformaldehyde by incubating for 15 minutes at room temperature. The cells were incubated with blocking buffer (5% bovine serum albumin (Sigma-Aldrich) in PBS) for 1 hour. Antibodies to vimentin (Abcam, mouse anti-human, polyclonal) and TNF-alpha (Abcam, rabbit anti-human, polyclonal) were added at a dilution of 1:200 in incubation buffer (10% blocking buffer, and 0.3% Tween 20 in PBS) and incubated at 4°C overnight shaker. Secondary antibody Alexa Flour 555 goat-anti-mouse and goat anti-rabbit (H+L) (Invitrogen) was used to visualise bound primary antibodies. Both secondary antibodies were diluted 1:500. Any excess secondary antibody was removed by washing 3 times with PBS. The cells were then stained with 4',6-diamidino-2-2phenylindole (DAPI) (Sigma-Aldrich) for 10 minutes in 1:20000 dilution and washed 3 times with PBS.

TaqMan gene expression assay

RNA was extracted from whole blood with PAXgene blood RNA kits according to the manufacturer's protocol (Qiagen Inc.). The amount and quality of RNA extracted was assessed with both a NanoDrop ND1000 (ThermoFisher) and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.), respectively. The following TaqMan Assays were used: Hs00234579_m1: MMP9 gene (sequence; GTACCGAGAGAAAGCCTATTTCTGCC) and Hs00247108_m1: PAD2 gene (sequence; GATGTCTACAGCGCGGCCCCAGCCG). Real time qPCR analysis was performed using BioMark Dynamic Array platform (Fluidigm) and pre-designed TaqMan Gene Expression Assays (Thermo Fisher). RNA (100 ng) was reverse-transcribed to cDNA using Superscript III First-Stand Synthesis according to manufacturer's instructions and then pre-amplified using TaqMan PreAmp Master Mix (Thermo Fisher). The pre-amplification reaction was performed at a final dilution of 0.05x original Taqman assay concentration. The thermocycling conditions were as follows: 1 cycle of 50°C for 15 min, 1 cycle of 70°C for 2 min, then 14 cycles of 95°C for 15 sec and 60°C for 4 min. Pre-

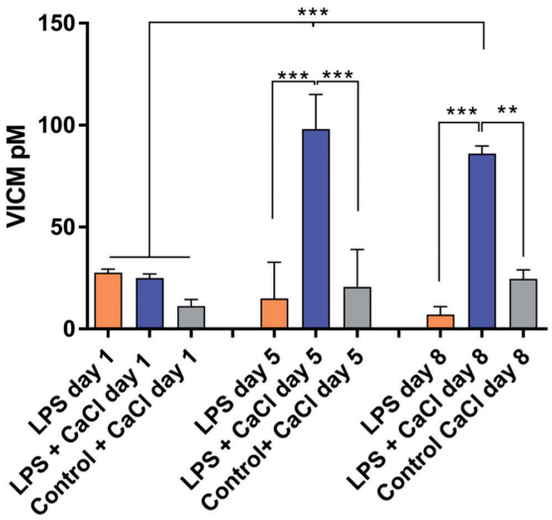

Fig. 2. Release of the citrullinated and MMP degraded fragment of vimentin (VICM) by macrophages under different conditions measured at day 1, 5 and 8 time points. LPS (100ng LPS treated macrophages), LPS + $CaCl_2$ (100ng LPS + 2mM $CaCl_2$ treated macrophages) Control (only $CaCl_2$ treated macrophages). Asterisk (*) indicate different levels of statistical significance; ***p<0.01, ***p<0.001*.

Table I. Patient demographics and baseline clinical characteristics*.

	Mavrilimumab			
	150 mg eow (n=79)	100 mg eow (n=85)	30 mg eow (n=81)	Placebo (n=81)
Age mean (range)	52.6 (24-73)	50.8 (22-76)	51.2 (27-79)	52.8 (25-76)
Female n (%)	67 (84.8)	70 (82.4)	70 (86.4)	75 (92.6)
Weight kg mean (SD)	75.9 (17.6)7	1.8 (16·2)	72.5 (152)	73 (15.2)
Body mass index, kg/m2 mean, (SD)	28.4 (6.2)	26.3 (5.3)	27.3 (5.1)	27.5 (5.1)
Years since RA diagnosis, mean (SD)	8.5 (6.9)	7.2 (6.5)	7.8 (6.6)	7.6 (7.2)
Methotrexate use, n	79	84	81	81
Dosage mg/week, mean, (SD)	14.5 (4.1)	15.1 (4.6)	14.6 (3.6)	15.0 (3.7)
ACPA-positive n (%)	61 (77.2)	63 (74.1)	66 (81.5)	59 (72.8)
DAS28 (SD)	5.7 (0.8)	5.9 (0.9)	5.7 (0.9)	5.8 (0.8)
Swollen joint count mean (SD)	15.7 (7.1)	16.8 (8.6)	17.8 (10.1)	14.4 (6.9)
Tender joint count mean (SD)	26.7 (11.4)	27.0 (14.2)	27.5 (14.0)	26.3 (11.3)

EOW: every other week; SD: standard deviation; RA: rheumatoid arthritis; mg: milligram; ACPA: anti-citrullinated protein antibodies; Mavrilimumab: anti-GM-CSFR-alpha monoclonal antibody.
*Full patient demographics can be reviewed in Burmester *et al*. 2016 (18).

amplified product was diluted 1:5 in TE buffer and analysed on Biomark 96.96 Dynamic Array according to the manufacturers' protocol. All samples were assayed in duplicate. Three endogenous reference genes (GUSB, TFRC, and UBC) were used to calculate delta Cts.

Statistical analysis

For RA clinical cohort, associations between baseline VICM serum levels and clinical scores were assessed by *Spearman*'s correlation tests and the significance was determined via the asymptotic *t* approximation followed by *Benjamini-Hochberg* (BH) adjustment. Student's *t*-test was performed on log-transformed data to investigate the difference in VICM serum levels at baseline between patients with higher or lower than median mTSS score. Changes in VICM serum levels were calculated as a function of time and treatment. Mavrilimumab induced changes were compared with VICM changes in the placebo arm by Student's *t*-test. The difference in VICM supernatant levels in the *in vitro* studies was calculated with a two-way ANOVA repeated measures and corrected for multiple comparisons with the Bonferroni test. GraphPad Prism 7.00 together with MedCalc was used for the statistical analysis.

Results

Macrophage characterisation

To evaluate the possibility of cellular toxicity of the treatments, the viability of the cells was measured by AlamarBlue® and the number of cells/cm^2 was calculated. MØ-L and MØ-LC had lower cell count/cm^2 compared to MØ-C (MØ-L=12,050cells/cm^2; MØ-LC=11,300cells/cm^2; MØ-C =14,100cells/cm^2). In general $CaCl_2$ treated macrophages decreased the cell metabolic output (Fig. 1A). MØ-LC had increased protein expression of CD68 and vimentin over the course of 5 days and compared to control MØ-C where the protein expression did not change from day 1–5 (Fig. 1B). Cell lysates from MØ-LC also showed bands (approximately 37 kDa) that indicate fragments of vimentin. Furthermore, MMP-2 and MMP-8 were expressed in both MØ-LC and MØ-C cells; however, MMP-2 was increased in MØ-LC cells over time compared to MØ-C cells. MMP-8 expression was the same in MØ-LC and MØ-C (Fig. 1B). Vimentin expression was also increased in MØ-LC, and vimentin demonstrated increased expression near the plasma membrane in MØ-LC compared to control MØ-C (Fig. 1C). The morphology of the MØ-LC had changed to a more spindle-like shape with protruding dendrites. The MØ-C was multinucleated and had a foam cell like appearance (Fig. 1C). Furthermore, TNF-alpha expression was also increased in the MØ-LC compared to control MØ-C (Fig. 1C).

VICM levels measured in the supernatant of LPS and Ca^{2+} treated macrophages vs. controls

MØ-LC showed a significant increase (*p<0.01*) in VICM levels in supernatants at day 5 and 8 compared to MØ-L and MØ-C at day 5 and 8 (Fig. 2). In addition, VICM levels of MØ-LC at day 5 and 8 were also significantly increased compared to day 1 (*p<0.001*) (Fig. 2). Furthermore, LPS or $CaCl_2$ treatments alone did not increase the VICM levels at day 5 or 8 significantly compared to the baseline measurement at day 1 (Fig. 2).

Patient characteristics

The phase IIb study included a total of 326 patients. The majority of the patients were female (n=282 (87%)) with mean age of approximately 52 years (range 22–79) and mean disease duration of 7.8 years. Patient demographics and baseline characteristics were similar between treatment arms (Table I).

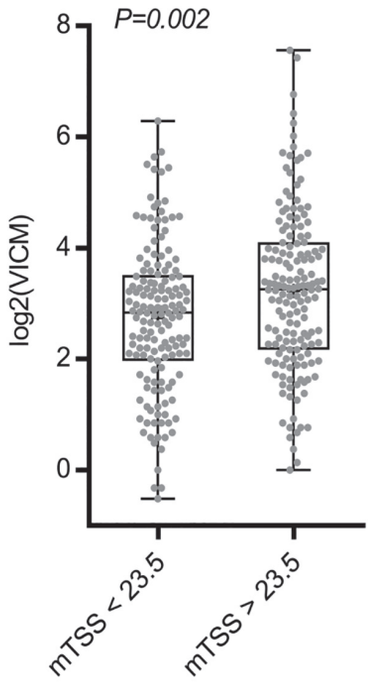

Fig. 3. VICM levels in RA patients at baseline with high mTSS score (>23.5) compared with patients with low mTTS score (≤23.5).

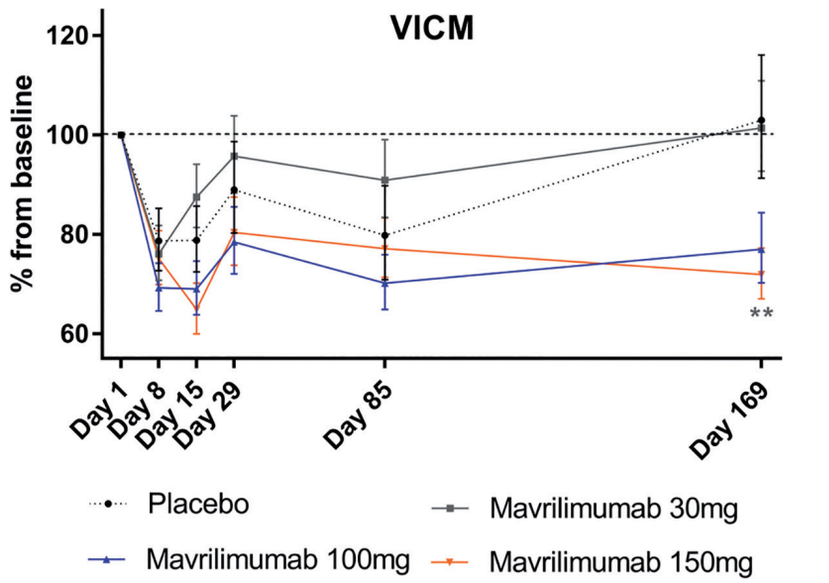

Fig. 4. VICM serum levels as % from baseline in RA patients over time for the placebo or mavrilimumab treated patients. Asterisk (*) indicate different levels of statistical significance; **$p<0.01$.

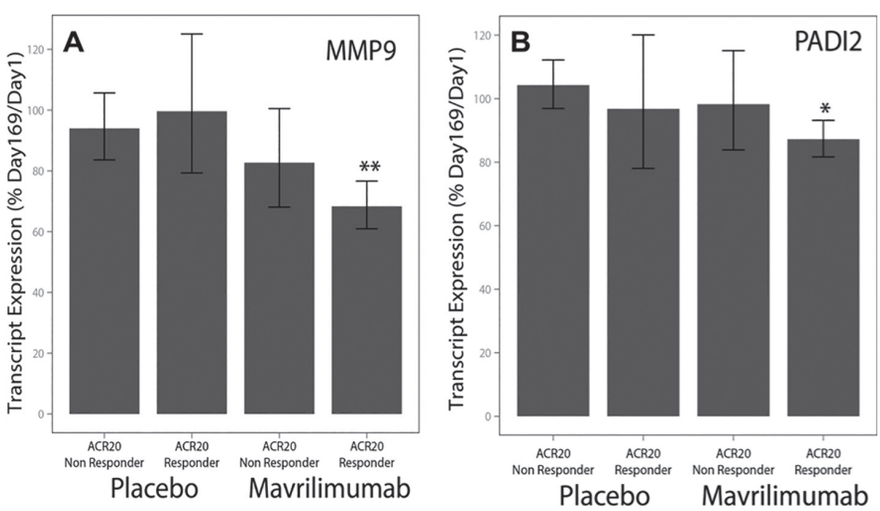

Fig. 5. Indicate suppression of A) MMP9, and B) PADI2 gene transcripts in mavrilimumab-treated ACR20 responders. Asterisk (*) indicate different levels of statistical significance;*$p<0.05$, **$p<0.01$.

VICM levels correlate with disease activity, joint space narrowing and erosion score

Since the GM-CSF is a cytokine essential for especially monocyte/macrophage survival, VICM was also longitudinally measured in the phase IIb study (NCT01706926) of mavrilimumab in RA. VICM serum levels at baseline correlated with DAS28 (r=0.13, $p<0.05$), modified Total Sharp Score (mTSS) (r=0.15, $p<0.01$), CRP (r=0.26, $p<0.01$), joint space narrowing (JSN) (r=0.15, $p<0.01$), and joint erosion (JE) (r=0.14, $p<0.01$). At day 169 VICM correlated with DAS28 (r=0.13, $p<0.05$), mTSS (r=0.16, $p<0.01$), JE (r=0.15, $p<0.05$), JSN (r=0.15, $p<0.05$) and CRP (r=0.29, $p<0.001$).

The association of VICM levels with high (> median) and low (≤ median) mTSS sharp score, at baseline, was also evaluated. Serum VICM levels were significantly higher in patients with high mTSS (>23.5 median mTSS) than in those with low mTSS (≤23.5 median mTSS) ($p<0.01$) (Fig. 3). Serum VICM levels did not, however, correlate with anti-citrullinated peptide antibodies (ACPA) serum levels at baseline or after treatment (data not shown).

VICM serum level is suppressed in mavrilimumab treated RA patients

It was observed that the VICM serum levels percentage from baseline to day 8 did decrease (>20%) in all groups including the placebo group. At day 15 only the groups treated with the highest doses mavrilimumab (100mg, 150mg) showed continued decrease (>30%) in VICM serum levels. At day 29 the VICM levels were decreased to >20% of baseline for the groups treated with the highest doses of mavrilimumab. At day 85 the groups treated with highest doses had a VICM serum level decrease of >20% of baseline measurements. However, the placebo and the 30mg group had <20% decrease in serum levels of VICM. At day 169, VICM serum levels decreased >30% of baseline for the group treated with 150mg of mavrilimumab ($p<0.01$), and the group treated with 100mg of mavrilimumab showed 25% decrease in VICM serum levels. In addition, the placebo group and patients treated with 30mg had a VICM serum level >1% of baseline at day 169 (Fig. 4). To support this, MMP-9 and PAD2 transcript expression were investigated. ACR20 responders to the mavrilimumab at day 169 of treatment had >25% suppression of the transcript

expression of MMP-9 ($p<0.01$) and >10% suppression of PAD2 ($p<0.05$), whereas no statistically significant changes of MMP-9 and PAD2 were observed in mavrilimumab-treated ACR20 non-responders and all patients treated by methotrexate alone (Fig. 5).

Discussion

The study at hand investigated the neo-epitope biomarker VICM and its relation as a biomarker of activated macrophages. Here, we demonstrated for the first time that VICM is a biomarker of activated macrophages, and VICM serum levels are significantly suppressed by mavrilimumab in RA patients.

In line with a previous publication demonstrating that vimentin is secreted by activated macrophages (22, 23), results presented in this study further contributes to the notion that VICM is a biomarker of activated macrophages. MMP-2 and MMP-8 have been shown, *in vitro*, to generate the VICM fragment. Here, we also demonstrated that the *in vitro* macrophages did express both MMP-2 and MMP-8. However, the expression of MMP-2 was more abundant than MMP-8. In addition, Western blots also revealed lower kDa bands, and these bands corresponds to the kDa of activated MMP-2 (24, 25) and MMP-8 (25). This indicates that activated macrophages can generate the VICM fragment. Thus, our findings support that VICM is a biomarker of activated macrophages, since the VICM fragment is only released from activated macrophages in the presence of high Ca^{++} levels. Previous studies have demonstrated that non-citrullinated forms of vimentin are secreted by macrophages *in vitro* (22) and *in vivo* (23). However, this is the first time that a citrullinated form of vimentin has been shown to be released by activated macrophages. Furthermore, the treatment of macrophages with LPS and $CaCl_2$ also showed increased TNF-alpha expression, which further indicates activation of the LPS and $CaCl_2$ treated macrophages.

Secondly, we showed that serum VICM levels were suppressed after the administration of 100 and 150 mg of the anti GM-CSFRα antibody, mavrilimumab, in RA patients at day 169 compared to baseline, and these doses were the only ones to be associated with VICM levels significantly different from baseline levels and to sustain a continuously suppressed VICM level in RA patients. Thus, by blocking the GM-CSFRα with mavrilimumab, macrophages production of VICM is suppressed and leads to decreased serum levels of VICM. VICM serum levels have previously been shown to be increased in autoimmune disease, including RA (16, 17), ankylosing spondylitis (17), and Crohn's disease (26). Common for these diseases are a macrophage involvement, and antibodies against citrullinated vimentin has been shown to be associated with the pathogenesis of RA (2, 3, 27). Based on these findings mavrilimumab could also be tested as potential treatment options for AS and Crohn's disease.

At baseline VICM was significantly elevated in patients with high mTSS sharp score compared to the patients with low baseline mTSS scharp score. This indicates that patients with increased joint erosion at baseline have increased activiated macrophages involvement. VICM was also found to correlate with mTSS and DAS28 disease activity indexes. Even though the correlation was modest for VICM and disease activity indexes (DAS28 and mTSS) correlation, VICM may still prove to have some clinical relevance in regards to disease activity in RA. Moreover, the fact that serum transcript levels of PADI2 were also suppressed by mavrilimumab demonstrates that the process of citrullination is diminished by blocking the GM-CSF receptor with mavrilimumab. Furthermore, the suppression of MMP-9 transcripts, together with the reduction in PADI2, indicates that mavrilimumab can suppress the MMP activity and PAD enzyme activity, which is also supported by the VICM suppression by mavrilimumab.

VICM has previously been shown to be suppressed by other effective biological treatments *e.g.* the IL-6 receptor blocker tocilizumab (16). IL-6 has been demonstrated to be released from fibroblasts and activated macrophages (28) and IL-6 promotes recruitment of monocytes leading to chronic inflammation (29). Thus, these data further strengthen the notion that VICM is a biomarker for activated macrophages.

A limitation is that MMP-8 has been shown to be highly expressed by neutrophils, and vimentin is not exclusively expressed by macrophages. Therefore it is possible that some of the VICM fragments also can be derived from neutrophil extracellular traps. Nevertheless, the citrullination process has been demonstrated several times in the literature to be highly associated with macrophages (14, 23, 30). Even though we consider it a strength for this study that VICM was measured in a controlled clinical phase IIb trial, this may also be a weakness. Clinical trials only represent a subset of RA patients. Therefore the results presented in this study should be validated in other cohorts with a bigger sample size.

In conclusion, we have demonstrated for the first time that VICM is a novel biomarker of activated macrophages *in vitro*, and the expression of MMP-2 and 8 by macrophages further support these data. Moreover, these findings were supported by the mavrilimumab study that demonstrated a 30% suppression of VICM, and mavrilimumab also suppressed PAD2 transcripts in mavrilimumab responders. Therefore, patients with a VICM profile may benefit from getting treatments targeting macrophages, *e.g.* mavrilimumab.

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