CD36 regulates macrophage and endothelial cell activation and multinucleate giant cell formation in Anti Neutrophil Cytoplasm Antibody vasculitis

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Abstract

Objective: To investigate CD36 in ANCA-associated vasculitis (AAV), a condition characterized by monocyte/macrophage activation and vascular damage.

Methods: CD36 expression was assessed in AAV patients and healthy controls (HC). The impact of palmitic acid (PA) stimulation on multinucleate giant cell (MNGC) formation, macrophage, and endothelial cell activation, with or without CD36 knockdown, was examined.

Results: CD36 was overexpressed on AAV patients' monocytes compared to HC, regardless of disease activity. AAV patients exhibited elevated soluble CD36 levels in serum and PR3-ANCA patients' monocytes demonstrated increased MNGC formation following PA stimulation compared to MPO-ANCA and HC. PA stimulation of macrophages or endothelial cells resulted in heightened CD36 expression, cell activation, increased macrophage migration inhibitory factor (MIF) production, and c-Myc expression, with attenuation upon CD36 knockdown.

Conclusion: CD36 participates in macrophage and endothelial cell activation and MNGC formation, features of AAV pathogenesis. AAV treatment may involve targeting CD36 or MIF.

Introduction

Anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitis (AAV) is a small vessel vasculitis, characterized by the presence of ANCA, antibodies that target the neutrophil and monocyte proteins myeloperoxidase (MPO) and proteinase 3 (PR3), leading to aberrant cell activation and endothelial damage through release of pro-inflammatory cytokines and proteases[1], . There are three main clinical subtypes, which differ in their pathophysiology and genetic predisposition, microscopic polyangiitis (MPA), Granulomatosis with polyangiitis

(GPA) and Eosinophilc granulomatosis with polyangiitis(EGPA) [2]. All result in systemic vasculitis with varied and multiple organ involvement [3], but GPA is additionally characterised by granuloma formation, most frequently in the upper and lower respiratory tract, the nose, sinuses and the eye [4]. We have recently shown in vitro that persistent PR3 stimulation of monocytes from GPA patients, leads to multinucleate giant cell (MNGC) formation and then mature multicellular granuloma, mediated by IL-6 and MCP-1 production. Additionally, we replicated this in vivo by developing a novel zebrafish model [5]. It appeared that GPA patients' monocytes expressed higher levels of PR3 binding partners making them susceptible to PR3 mediated MNGC formation.

CD36, is a transmembrane glycoprotein scavenger receptor that plays a role in various cellular processes, including modulation of inflammatory and immune responses and angiogenesis[6]. It is expressed on multiple cells including monocytes, macrophages and vascular endothelial cells [7]. Increased levels of CD36 have been observed in several chronic inflammatory conditions, such as obesity, diabetes, atherosclerosis, and liver disease [8,9]. Soluble CD36 (sCD36), the extracellular portion of the CD36 receptor that is released into the bloodstream [10], has been implicated in modulating inflammation and is associated with various inflammatory conditions [11]. In addition, CD36 has been shown to be an important mediator for macrophage fusion, leading to MNGC formation through lipid, and specifically phosphatidylserine (PS), binding [12]. Intriguingly, apoptotic neutrophils from GPA patients display greater levels of surface PR3, phospholipid scramblase-1 and Annexin 1- involved in PS binding which leads to reduced macrophage clearance of the cells, potentially allowing them to persist and stimulate macrophages to form giant cells [13]. Taken together these data suggest that CD36 may be another important mediator of tissue inflammation and damage in AAV, but has been understudied, so we sought to investigate the role of CD36 in AAV.

Methods

Patients and controls

AAV patients with active disease or in remission were identified through the Royal Free Vasculitis clinic. Additional samples from patients with active disease were obtained from the University Hospital Birmingham. The disease was classified according to the Chapel Hill Consensus Conference (CHCC) diagnostic criteria, and following consent, they underwent venesection [14]. Patient demographics, clinical characteristics and investigations including ANCA reactivity were documented from electronic records. Disease activity, scored by Birmingham Vasculitis Activity Score (BVAS) was calculated using the BVAS 3 calculator. Healthy controls were identified from laboratory staff and healthy volunteers.

PBMC Isolation

Peripheral blood mononuclear cells(PBMC) were isolated using lymphoprep according to standard protocols [15]. Monocytes were purified through a 2-hour adhesion step.

THP1/macrophages MNGCs formation

The THP1 cell line (purchased from the European Collection of Authenticated Cell Cultures) was treated with phorbol 12-myristate 13-acetate (PMA) for 2 days, followed by treatment with 50 μM palmitic acid (PA) for an additional 5 days to stimulate multinucleated giant cell (MNGC) formation. MNGCs were verified using a protocol similar to the one described in a previous publication by our group [5]. MNGC slides were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde. Wheat germ agglutinin (10 μg/mL) was added for membrane staining, followed by incubation for 10 minutes. After washing the cells three times with PBS, Phalloidin conjugate working solution (1X, 100 μL) was added and

incubated at room temperature for 20 minutes. The cells were stained with DAPI, and images captured using a fluorescence microscope.

MNGCs Giemsa Staining and Fusion Index Calculation

Cultured THP1/macrophage samples were stained using a modified Giemsa stain [16]. The stained samples were scanned using a light microscope and analyzed using ImageJ software (Fiji) according to previously published methods [5]. Briefly, each sample was scanned at a magnification of x100, converted to an 8-bit format, and rendered in grayscale. The images were then converted to black and white, and a binary "watershed" function was applied to distinguish between pixel values in areas of aggregation. This allowed for the precise measurement of the percentage of fused cells per surface area.

CD36 Knockdown and Supernatant Cytokine Detection in THP1/Macrophages and Human Microvascular Endothelial Cells (MEC)

Human CD36 siRNA was purchased from Horizon. The CD36 knockdown process followed the siRNA transfection protocol. Briefly, siRNA was added to serum-free medium, mixed with DharmaFECT transfection reagent, and incubated for 20 minutes at room temperature. Antibiotic-free complete medium was added, and cells were incubated at 37°C for 48 hours.

Supernatant cytokine detection Human Cytokine Array Kit was purchased from R&D Systems. The membranes were incubated with a detection antibody cocktail and sample supernatants for 1 hour according to manufacturers' instructions. In addition supernatant MIF levels were measured using the Human MIF Quantikine ELISA Kit purchased from R&D Systems according to manufacturers' instructions.

MIF antagonist intervention on cells

The MIF antagonist, ISO-1 [17], was acquired from Sigma-Aldrich. Both macrophages and vascular endothelial cells were incubated with the MIF antagonist for 24 hours before conducting the experiments.

Mitogen-Activated Protein Kinase (MAPK) Transcription Factor Assay

The MAPK transcription factor assay kit was purchased from Cambridge Bioscience and used in accordance with the manufacturer's instructions. The c-Myc inhibitor (Calbiochem) was obtained from Sigma-Aldrich.

THP1/Macrophage Migration Assay via Giemsa and CFSE Staining in the Co-Culture Model

For Giemsa staining, MEC were cultured alone with palmitic acid (PA) and CD36 knockdown for two days, and THP1/macrophages were cultured on the top chamber in the co-culture model. THP1/macrophage inserts were stained with Giemsa. Non-migrated cells on the top of the inserts were removed by scraping with a cotton swab, and the migrated cells on the bottom inserts were counted under a microscope [18].

For carboxyfluorescein succinimidyl ester (CFSE) staining, THP1/macrophages were cultured in CFSE for 20 minutes, and then the CFSE was quenched by adding the original cell culture medium. Afterwards, THP1/macrophages were co-cultured with MEC previously treated with PA with/without CD36 knockdown. After 2 days, migrated cells in the bottom transwell were collected, and fluorescent cells were analyzed by flow cytometry [19].

Real-Time PCR Analysis

RNA was extracted and synthesized into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Quantitative real-time PCR (qPCR) was performed on a

LightCycler 96 system (Roche) using the cDNA and SYBR Green PCR Master Mix (Thermo Fisher). The primers used for amplification are shown in the supplementary table 2.

Flow Cytometry

Cells were resuspended in flow cytometry staining buffer (R&D Systems) and incubated with 10% BSA (Sigma) for approximately 20 minutes. Conjugated anti-CD36 antibody (Abcam) was added, followed by the addition of conjugated CD14 (BioLegend) and CD16 (BD) antibodies. After incubation for 30 minutes at room temperature, the cells were washed three times with FACS staining buffer. VCAM-1 (Abcam) staining was performed after permeabilization of the cells. The samples were analyzed using a Fortessa flow cytometer (BD Biosciences), and the results were analyzed using FlowJo software (BD Biosciences).

Immunohistochemistry and Immunofluorescence

For immunohistochemistry, sections were deparaffinized and subjected to antigen retrieval using EDTA and microwave heating. The sections were incubated with mouse anti-CD36 antibody (Novus) overnight and developed with ImmPRESS Polymer Reagent and ImmPACT DAB Substrate, Peroxidase (HRP) (Vector).

For immunofluorescence, after blocking with 5% normal goat serum, sections were incubated with anti-CD36, and either anti-CD68 or anti-CD163 antibodies (1:500) (Proteintech) before capturing images under a confocal microscope.

Quantification of CD36 expression involved analyzing the intensity of the brown-coloured precipitate in immunohistochemistry and the fluorescence intensity in immunofluorescence images using ImageJ software (https://imagej.net/ij/). The threshold for positive staining was established by referencing the background signal observed in one healthy control sample,

which was then set to zero. Using the same settings, we then quantified the staining in the remaining controls' and patients' samples. We performed whole kidney analysis and in addition used a region of interest to quantify only glomerular CD36 expression.

Soluble CD36 Detection

Serum samples were tested using the Human CD36/SR-B3 DuoSet ELISA kit (bio-techne, cat#DY1955-05) according to manufacturers' instructions.

Free Fatty Acid and Oxidised-LDL Analysis

Serum samples underwent were tested using the Free Fatty Acid Assay Kit (abcam, cat# Ab65341) and the Ox-LDL assay (abcam, cat# ab285269) according to manufacturers' instructions.

Statistical Analyses

All experiments were performed in triplicate. Data were analyzed using t-test when comparing two groups and one-way ANOVA when comparing multiple groups, using GraphPad Prism v10. All data are expressed as median ± range. A p-value less than 0.05 was considered statistically significant, and the significance level was indicated in the figures as follows: *$p<0.05$, **$p<0.01$.

Study Approval

The research described was conducted in accordance with the Declaration of Helsinki and approved by the NHS Research Ethics Committee (05/Q0508/6) and (10/H1102/77 NRES London).

Results

Patients

We recruited 39 AAV patients from the Royal Free Hospital London, who had samples tested in different assays. One patient had 2 samples collected longitudinally during acute disease and remission. Twenty were women, and nineteen were men, with a median age of 62 years (range 23-85 years). There were a total of 20 GPA patients, of which 19 expressed PR3-ANCA, and one MPO-ANCA. There were 17 MPA patients, all with MPO-ANCA, and 2 EGPA patients, both expressing MPO-ANCA. Among the 39 patients, 13 had active disease (median BVAS 6, range 1-12), and 26 were in remission (BVAS 0). Healthy controls were recruited from staff and volunteers and had a median age of 60 years (25-71). A validation cohort of 45 patients from Birmingham University Hospital were included for sCD36 measurements. The data are summarized in Supplementary Tables 1A-B.

CD36 is expressed in AAV kidney biopsies on macrophages

Using kidney biopsies from patients with GPA or MPA and healthy kidney donors CD36 expression was assessed using immunohistochemistry and immunofluorescence to co-localise with the macrophage marker CD68 and CD163. Immunohistochemical and immunofluorescence analysis demonstrated a significant upregulation using image analysis of CD36 expression in both GPA (PR3-ANCA positive) and MPA (MPO-ANCA positive) patients' samples compared with the healthy donor kidney tissue (Figure 1 A-C) (both $p<0.01$), with predominant glomerular and periglomerular staining. Using double immunofluorescence staining, we confirmed that CD36 co-localized with the macrophage marker CD68 and in some cells with the M2 macrophage marker CD163 (Figure 1A-B).

Increased CD36 expression on circulating monocytes of AAV patients

The expression of CD36 in different monocyte subsets was investigated in AAV patients

(n=18) and healthy controls (HC) (n=11). Monocytes were classified according to their expression of CD14 and CD16 into classical, intermediate, or non-classical subsets (Supplementary Figure 1). CD36 expression (Mean fluorescence intensity, MFI) was significantly increased on classical and intermediate monocytes from both PR3 and MPO-ANCA positive vasculitis patients during active disease and disease remission compared to HC, with no difference on non-classical monocytes (Fig 2A-C, E); Classical monocytes in HC median (range): 46971 (22832-76878) vs. active patients 68948 (52972-88343), $P<0.05$ vs. patients in remission 75239 (35769-110473), $P<0.01$, and in intermediate monocytes HC: 44186 (18864-61112) vs. active patients 64480 (55634-74052), $P<0.05$ vs. patients in remission 57069 (37722-130121), $P<0.01$. CD36 expression did not differ according to ANCA subtype (Fig 2D); Healthy controls, PR3-ANCA patients, and MPO-ANCA classical monocytes: 46971 (22832-76878) vs. 70286 (35769-110473), $P<0.05$ vs. 57048 (48529-100929), $P<0.05$ respectively, and in intermediate monocytes: 44186 (18864-61112) vs. 55634 (41202-130121) $P<0.01$, vs. 64480 (37722-91679), $P<0.05$. While MFI was increased on patients' cells, there was no significant difference in the percentage of cells expressing CD36 between patients and HC in any of the monocyte subsets, when analysed according to disease activity or ANCA subtype (Figure 2 F-G).

Soluble CD36 expression, free fatty acid and oxidised LDL levels in AAV sera

In addition to our cohort of patients we added a validation cohort for sCD36 level measurement. This consisted of 45 samples taken at the time of acute disease presentation which were analysed using the same methodology. We combined these two cohorts as there was a large variance in the levels detected. Increased levels of soluble CD36 (sCD36) were found in the sera of both MPO-and PR3- ANCA patients, compared with HC, (Figure 3A).

PR3-ANCA patients' sCD36 levels median (range): 146 (0-4570) pg/ml, MPO-ANCA: 70 (0-8815), and HC: 0 (0-305.5) (HC vs PR3-ANCA $P<0.01$, HC vs MPO-ANCA $p<0.05$). However, like the level of surface expression, these did not differ between acute disease (levels: 43 (0-8815) pg/ml) and disease remission (levels: 165 (0-501.7) pg/ml, (Fig 3B). Moreover, we found no significant correlation between disease activity assessed by BVAS and sCD36 levels (data not shown). Finally, we confirmed that PA or TNF- α stimulation of macrophages and MEC augments its own cell surface expression and leads to increased sCD36 production, which is attenuated by CD36 knockdown (Supplementary Figure 2).

We investigated levels of CD36 ligands in the sera, measuring free fatty acid and oxidised LDL levels. Whilst we found no statistical difference in circulating free fatty acid levels between AAV patients and age matched controls, AAV median (range):0.03nmol/mcl (0.005-0.048), HC 0.01 (0.001- 0.04), $p=0.091$ (Figure 3C), we found higher levels of oxidised LDL in both acute and remission samples and in both PR3- and MPO- ANCA samples (Figure 3 D-E) compared with HC; Active AAV 226.6 ng/ml (36.4-327.2), Remission AAV 124.3 (8.0-324.2) $P<0.05$; MPO-ANCA: 151.1 (8.02-324.2), PR3-ANCA Median: 124.3 Range: (10.31-327.2), Control group: 32.82 (0-208.4), $P<0.05$.

Increased MNGC formation and MIF production in AAV patients following CD36 stimulation

Monocytes were isolated from GPA (PR3-ANCA positive, all in remission) patients (n=6) and HC (n=6) following venesection, PBMC isolation, and a two-hour adhesion step. Subsequently, they were stimulated with PA for an additional three days to induce MNGC formation. At the end of the experiment, supernatants were collected and tested for levels of MIF by ELISA. A significantly elevated MNGC fusion index was observed in AAV patients (Figure 4A) when compared to HC (Figure 4B); In HC fusion index median (range) 1.42

(1.14-1.62), while AAV patients had a fusion index of 2.83 (2.29-3.09), P<0.01. Cell supernatants displayed higher levels of MIF concentration in AAV patients compared to the control group (Figure 4D); HC MIF level 6.92 ng/ml (5.69-7.85), AAV patients MIF level 7.95 ng/ml (7.54-9.65), P<0.05.

PA stimulation of THP1-macrophages promotes formation of multinucleated giant cells (MNGC) mediated by CD36, MIF and c-Myc.

THP1-matured macrophages were stimulated for five days with PA and cell morphology assessed (Figure 5A). Following PA stimulation macrophages upregulated M1 markers CD86, CD80 and HLA-DRB (Figure 5D-F) and downregulated the M2 marker CD206 (Figure 5G). In addition, fluorescence microscopy following staining of the cells with combined nuclear, membrane and cytoskeletal stains, demonstrated clear MNGC contained within a single membrane (Figure 5B-C). The extent of MNGC formation was quantified using previously described methods [5] based on image analysis. Further experiments using different doses of PA were performed to identify the optimal stimulus dose, which increased the fusion index up to 50 μM (Figure 5H) as well as duration of stimulation which yielded no greater degree of giant cell formation beyond day 5 (Figure 5I). However, in cells preincubated with CD36 siRNA, which resulted in a 37% reduction in CD36 expression (Supplementary figure 3), MNGC formation was significantly reduced (Figure 5J). Furthermore, we compared MNGC formation following either PA or PR3 stimulation, as we had previously shown this to significantly stimulate MNGC formation in AAV patients and HC and demonstrated equal effect of both stimuli (Figure 5K).

As we had previously demonstrated a dependence on IL-6 for MNGC formation following PR3 stimulation of monocytes, we tested cytokines produced following PA stimulation. We found no increase in IL-6 but observed a significant production of macrophage inhibition

factor (MIF), which was confirmed using an MIF-specific ELISA (Figure 5L and Supplementary figure 4), while MIF production following PA stimulation of macrophages was inhibited by CD36 siRNA preincubation (Figure 5L). MIF concentration, median(range), in unstimulated cells: 0.150 (0.14- 0.16) ng/ml, PA stimulated: 0.47 (0.46-0.51) ng/ml, $P<0.01$, and PA stimulated with CD36 siRNA: 0.436 (0.43-0.45) ng/ml, $p<0.01$). To further confirm the importance of MIF in MNGC formation, we co-incubated macrophages with PA and an inhibitor of MIF, which resulted in a 15.7% reduction in MNGC formation. Notably, this reduction was most significant when inhibitor was added on day 2 of a 7-day incubation but still had a noticeable effect when added toward the end of culture on day 5 (Figure 5M). Finally, we used a transcription factor assay and found that following PA stimulation there was an upregulation of the MAPK transcription factor c-Myc which was attenuated by CD36 siRNA (Supplementary figure 5). Inhibition of c-Myc using a specific antagonist demonstrated reduced macrophage activation, MNGC formation and MIF production (Supplementary figure 5).

Endothelial cell activation by PA is mediated through CD36 and through bidirectional macrophage interactions

Stimulation of MECs with PA resulted in an increase in surface VCAM-1 expression (Figure 6A-B) and MIF production (Figure 6D), both of which were attenuated by CD36 knockdown (Figure 6C-D). In unstained cells, the VCAM-1 MFI: 45 (43-48); in unstimulated cells: 1350 (1299-1420); in PA stimulated: 2487 (2379- 2540), $p < 0.01$; and in PA stimulated with CD36 knockdown: 1690 (1683, 1751), $p < 0.01$. Supernatant MIF levels in unstimulated cells, PA-stimulated cells and PA stimulated with CD36 knockdown 0.15 (0.14-0.16) vs. 0.47 (0.46-0.51), vs. 0.436 (0.43-0.45) respectively, $p < 0.01$. However, there was no significant difference in percentage of CD36 positive cells between control and experimental

groups (Figure 6D).

We next sought to investigate the interaction between activated macrophages and MEC using a co-culture model. Macrophages were cultured with 50uM PA or PA after CD36 knockdown for 48 hours and added to MECs for an additional 48 hours. MEC activation was assessed by VCAM -1 expression. Compared to MEC alone, those coincubated with PA-treated macrophages expressed higher levels of VCAM-1. This upregulation was reduced if the macrophages were pre-treated with CD36 siRNA (Figure 6E). In unstained cells VCAM-1 MFI was 782 (769-868); in stained unstimulated cells, 9875 (9654-10990), $p < 0.01$; in PA stimulated cells, 18082 (17892-18320), $p < 0.01$; and in PA-stimulated cells with CD36 knockdown 16124 (15896-16632), $p < 0.01$. Similar to MEC culture alone, there was no significant difference in the percentage of CD36 positive cells in the co-culture model (Figure 6H).

Performing the experiment in reverse, we activated MEC using PA and incubated the cells with unstimulated macrophages. We assessed CD86 expression as a marker of macrophage activation and showed that macrophages co-cultured with PA-treated MECs significantly increased CD86 expression, which was reduced when the MECs were preincubated with CD36 siRNA (Figure 6G). Unstimulated MEC cocultured macrophage CD86: 0.9918 (0.90-1.11); PA stimulated cells: 1.78 (1.46-2.0), $p < 0.01$; PA -stimulated with CD36 knockdown: 1.59 (1.33-1.66), $p < 0.01$.

Finally, we showed that PA treated MEC promote macrophage migration towards them, by labelling macrophages with CFSE and separating them from the activated MEC in a co-

culture chamber. There was an increase in the percentage of CFSE-positive macrophages that migrated from the insert to the MEC side of the insert with PA-treated MEC (Figure 6I) compared to untreated MEC (Figure 6H). However, this increase was attenuated in the presence of MEC CD36 knockdown (Figure 6J). Unstimulated MEC percentage CFSE positive macrophages: 7.01 (6.80-7.15); PA-stimulated cells: 13.20 (12.8-14.4), $p < 0.01$ and PA-stimulated with CD36 knockdown: 8.32 (7.89-8.81), $p < 0.01$. These data demonstrate that in the presence of PA, both MECs and macrophages become activated in a CD36-mediated manner, promoting macrophage recruitment and induction of proinflammatory phenotypes in each other.

Discussion

Our study provides valuable insights into the potential role of CD36 in the pathogenesis of AAV, highlighting its involvement in macrophage activation, endothelial cell dysfunction, and MNGC formation, key components in AAV pathogenesis.

CD36 and sCD36 have been proposed as potential biomarkers in various inflammatory diseases. For instance, CD36 facilitates the uptake of oxidized low-density lipoprotein (LDL) by macrophages, leading to foam cell formation and the initiation of atherosclerotic lesions [20,21]. Increased CD36 expression on monocytes and macrophages has been observed in atherosclerosis patients and correlates with disease severity [22]. Furthermore, elevated CD36 expression has been reported in adipose tissue, skeletal muscle, and liver of individuals with obesity [23], and metabolic syndrome [24]. CD36 expression is also upregulated in the inflamed intestinal mucosa of inflammatory bowel disease patients [25]. Enhanced CD36 levels on macrophages and epithelial cells are associated with increased pro-inflammatory cytokine production and impaired resolution of inflammation [26]. Additionally, elevated CD36 expression has been detected in synovial tissue and macrophages of rheumatoid arthritis

patients [27]. Altered CD36 expression on immune cells has also been observed in septic mice and correlates with disease severity [28].

The upregulation of CD36 expression on monocytes, particularly classical and intermediate subsets, in PR3- and MPO-ANCA AAV patients, irrespective of disease activity, suggests that CD36 may not serve as a disease biomarker in AAV, but could mediate some persistent inflammatory responses in those patients and lead to a positive cycle of monocyte/macrophage activation and recruitment, as well as contributing to the accelerated atherosclerosis found in these patients [29].

Soluble CD36 (sCD36), represents the cleaved or shed form of the CD36 found in the bloodstream [30]. Increased levels of sCD36 were observed in the sera of AAV patients. In vitro experiments revealed elevated sCD36 levels in THP1/macrophages and MEC upon stimulation with PA or TNF-α. Interestingly, sCD36 has been implicated in several conditions, with elevated levels in patients with atherosclerosis [31], obesity [32] and type 2 Diabetes [33], as well as in systemic lupus erythematosus [34] and rheumatoid arthritis [35]. Our study confirms that CD36 knockdown decreases sCD36 expression, consistent with previous findings[36]. Again, we found no differences in levels in acute disease or remission making it an unhelpful disease biomarker. However, many other proinflammatory factors such as IL-17 or calprotectin have been shown to remain above normal levels in AAV patients during remission, highlighting the discrepancy between clinical and immunological remission.

Our study provides evidence that stimulation of macrophages and endothelial cells with palmitic acid induces an increase in CD36 expression, and sCD36 release, confirming the role of CD36 in the cellular response to PA, shown previously in various cell types [37,38 39,40].

The upregulation of CD36 expression observed in our study was accompanied by the

polarization of macrophages towards the M1 phenotype, and activation of endothelial cells, accompanied by the significant production of MIF. Previous research has indicated that PA can drive macrophages towards the M1 phenotype through Toll-like receptor 4 (TLR4) activation [41], while CD36 can itself modulate TLR4 signalling [42], and MIF production. Importantly, our study revealed that PA stimulation led to an increase in multinucleate giant cell formation. This phenomenon may be attributed to the ability of PA to induce changes in cell membrane composition and fluidity, which can promote cell fusion [43].

Moreover, our study uncovered a potential interplay between CD36, MIF, and c-Myc. Following PA stimulation, there was aCD36 dependent increase in c-Myc expression, This may be due to the involvement of CD36 in lipid metabolism and fatty acid uptake [44] , themselves associated with c-Myc expression [45], and may be linked by the mTOR signalling pathway [46]. Inhibiting either c-Myc or MIF showed a decrease in the expression of the other, confirming a potential regulatory relationship between them and potential feedback loops that regulate their expression [47,48]. These findings highlight CD36 as a potential modulator in the regulatory network connecting MIF and c-Myc.

Furthermore, our study demonstrated that microvascular endothelial cells can be activated through a CD36 dependent mechanism, confirming findings in human umbilical vein endothelial cells [49] potentially promoting endothelial dysfunction and inflammation [50].

The interaction between macrophages and microvascular endothelial cells plays a crucial role in the pathogenesis of AAV, and CD36 appears to facilitate enhanced macrophage activation and migration and endothelial cell activation, in a positive feedback loop, potentially further amplifying the inflammatory response.

Limitations of the study include our use of THP1-derived macrophages, although we did

confirm this with primary monocytes with similar results. In addition, we only used palmitic acid as a CD36 stimulus, and do not know the impact that other agonists such as oxidised LDL may have.

Conclusion

This study underscores the significance of CD36 in the pathogenesis of AAV and its contribution to various inflammatory processes, such as macrophage activation, MIF production, adhesion molecule expression, and cell-cell interactions. The findings suggest that interventions targeting CD36 or its downstream signalling pathways may hold promise as potential therapeutic strategies for AAV.

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Figure Legends

Figure 1: Increased CD36 Expression in kidney biopsies from patients with GPA and MPA

Immunohistochemical and immunofluorescent staining of CD36 in revealed a marked increase in CD36 expression in the renal tissue of patients with granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) compared to healthy donor kidney tissue (A-C, F-G). Semi-quantitative assessment of CD36 expression using ImageJ software confirmed the significant upregulation observed in GPA and MPA samples using both

immunohistochemical and immunofluorescent staining in whole kidney (D) and in glomeruli only (E). The data, presented as median ± range, were obtained from three independent measurements across the tissue sample for both patient and healthy donor tissues. Statistical analysis using one-way ANOVA demonstrated a highly significant difference (**P < 0.01) in CD36 expression levels between the MPA and GPA patient samples and control samples. Immunofluorescent staining revealed overlapping staining patterns of CD36 (green) and CD68 (red)(F), or CD36 (red) and CD163 (green) (G), indicating that CD36 expression is predominantly observed in CD68-positive macrophages and to a large extent, but not exclusively, in CD163 positive cells. Some tubular autofluorescence is seen.

Figure 2: Increased CD36 expression in classical and intermediate monocytes in AAV patients

CD36 expression in classical monocytes (A), intermediate monocytes (B), and non-classical monocytes (C) was analyzed by flow cytometry. The mean fluorescent intensity (MFI) and percentage of CD36 positive cells were calculated using FlowJo software for the active and remission groups (D, F) and PR3-ANCA and MPO-ANCA groups (E, G). The study included a total of n=5 active vasculitis patients, n=13 remission vasculitis patients, n=7 PR3-ANCA positive patients, n=11 MPO-ANCA positive patients, and n=11 healthy controls. Data are presented as median ± range. Statistical significance was determined using one-way ANOVA. *P < 0.05 and **P < 0.01 indicate significance compared to classical monocytes in the healthy control group, while $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01 indicate significance compared to intermediate monocytes in the healthy control group (D and E).

Figure 3: Increased soluble CD36 (sCD36) levels, free fatty acid and oxidised-LDL in AAV patients

Serum samples from healthy controls, MPO- and PR3-ANCA vasculitis patients, in remission and during active disease were analyzed using a commercial ELISA. sCD36 levels were significantly higher in both PR3-ANCA and MPO-ANCA patients compared to HC (A), while they appeared to be equally elevated in active disease or disease remission (B). The data are presented as median (A, B), analyzed using one-way ANOVA. Statistical significance is indicated as **$P < 0.01$ and *$P < 0.05$ compared to the control group (n=11 for healthy controls, n=40 for MPO-ANCA, n=34 for PR3-ANCA, n=20 for the remission group, and n=54 for the active group). Levels of serum free fatty acid in AAV cohort compared with HC were not significantly different (C) but levels of oxidised-LDL in AAV patients according to disease state (D) and ANCA subtype (E) showing statistically elevated levels compared to HC (both $p<0.05$)

Figure 4: AAV patients exhibited enhanced MNGC fusion and elevated MIF expression

Monocytes isolated from PBMC extracted from AAV patients (n=6) or healthy controls(n=6) were incubated for three days with or without PA, and Giemsa staining was performed. Quantification of cell fusion was assessed and analyzed using ImageJ. Compared to the healthy control group (A), there was a visible increase in MNGC from AAV patients' stimulated with PA (B), which on quantification was statistically increased(C) ($P < 0.01$). Cell supernatants were collected and MIF concentrations were measured by ELISA (D), revealing elevated MIF levels produced by AAV patients' cells compared to HC ($P < 0.05$).

Figure 5: PA stimulation of THP1-macrophages promotes MNGC formation and is mediated by CD36.

THP1-macrophages stimulated with PA for 5 days, were stained with Giemsa (A). Immunofluorescence demonstrated clear MNGC surrounded by a single cell membrane (B-C)

(nuclear staining with DAPI (blue), cytoskeletal staining with phalloidin (green), and membrane staining with wheat germ agglutinin (red)). Magnification (A) x200, (B-C) x400. Cells were also analysed for macrophage (M1) markers CD86, CD80, and HLA-DRB9, (D-F), and (M2) CD206 (G). The optimal dose and duration of PA stimulation for maximal fusion was investigated (H-I). Cells pre-treated with CD36 siRNA showed attenuated responses(J),but there was no difference following stimulation with PA or PR3 (previously shown to be fusinogenic) (K). PA stimulation led to increased supernatant MIF production, attenuated by CD36 knockdown (L). The addition of a MIF antagonist on day 2 or day 5 of a 7 day culture reduced MNGC formation (M). The data are presented as median ± range. **P < 0.01 vs. control group (D-G, J-M), $^{\#\#}$P < 0.01 vs. PA group (J, L), **P < 0.01 vs. day 0 (H), **P < 0.01 vs. 0 um PA (I), $^{\#}$P < 0.05 vs. PA group, and $^{\&\&}$P < 0.01 vs. MIF antagonist (5th day) group (M), all by one-way ANOVA.

Figure 6: Endothelial cell activation by PA is mediated by CD36

Following MEC stimulation with PA for 48 hours VCAM-1 expression increased (% cells and Mean fluorescence intensity (MFI))(A). Preincubation with CD36 siRNA for 24 hours attenuated VCAM-1 upregulation (B-C), **P < 0.01 vs. unstained group, $^{\#\#}$P < 0.01 vs. control group, and $^{\&\&}$P < 0.01 vs. PA group (C), one-way ANOVA. Following PA stimulation, supernatant MIF levels were elevated compared to unstimulated cells, and attenuated with prior CD36 siRNA (D). Similarly, MEC VCAM-1 was upregulated when MEC were cocultured with PA stimulated macrophages and was dependent on CD36 expression (E); **P < 0.01 vs. unstained group, $^{\#\#}$P < 0.01 vs. control group, and $^{\&\&}$P < 0.01 vs. PA group, one-way ANOVA. Conversely, MECs stimulated with PA, co-cultured with macrophages for two days promoted increased macrophage CD86 expression, compared to unstimulated MEC, while this was attenuated by prior CD36 siRNA (G); **P < 0.01 vs.

control group, and $^{\#\#}P < 0.01$ vs. PA group, one-way ANOVA. Macrophage migration towards PA-treated MEC was increased compared with unstimulated MEC (H. I, K), and reduced with CD36 siRNA (J). All data are presented as median ± range. $^{**}P < 0.01$ vs. control group, and $^{\#\#}P < 0.01$ vs. PA group, one-way ANOVA.