RHEUMATOLOGY

Rheumatology 2022;61:834–845
doi:10.1093/rheumatology/keab413
Advance Access publication 11 May 2021

Original article

Danger-associated molecular pattern molecules and the receptor for advanced glycation end products enhance ANCA-induced responses

Theresa H. Page [1], Derick Chiappo[1], Francesca Brunini[1,2], Josep Garnica[1,3], Jack Blackburn[1], Fayaz Dudhiya[1], Maria Prendecki[1], Stephen P. McAdoo[1] and Charles D. Pusey[1]

Abstract

Objectives. The pro-inflammatory activities of the calgranulins and HMGB1 can be counteracted by sRAGE, the soluble form of their shared receptor. To understand the role of these molecules in AAV and their potential as therapeutic targets we have studied (i) the relationship between these DAMPS and disease activity; (ii) the expression of RAGE and sRAGE in biopsy tissue and peripheral blood; and (iii) the effect of these molecules on ANCA-mediated cytokine production.

Methods. We examined circulating levels of calgranulins (S100A8/A9 and S100A12), HMGB1 and sRAGE by ELISA. RAGE was examined in AAV kidney and lung biopsies by immunohistochemistry and RAGE expression was monitored in peripheral blood by qPCR. *In vitro*, the effect of co-stimulating PBMC with ANCA and S100A8/A9 on cytokine production was studied by ELISA.

Results. We found significantly raised levels of calgranulins and HMGB1 in active AAV regardless of clinical phenotype (PR3+/MPO+ AAV). Levels of calgranulins showed significant correlations with each other. RAGE protein and message was raised in peripheral blood and in cells infiltrating kidney and lung biopsy tissue, while sRAGE was lowered. Furthermore, ANCA-mediated production of IL-8 from PBMC was significantly enhanced by the presence of S100A8/A9 in a RAGE/TLR4-dependent manner.

Conclusions. Raised circulating calgranulins provide a good marker of disease activity in AAV and are unlikely to be counteracted by sRAGE. Increased RAGE expression in AAV indicates receptor stimulation in active disease that may exacerbate ANCA-induced cytokine production. Targeting the RAGE pathway may provide a useful therapeutic approach in AAV.

Key words: ANCA-associated vasculitis, DAMP, RAGE, cytokines

BASIC SCIENCE

Rheumatology key messages

- Circulating S100A8/A9, S100A12 and HMGB1 levels are raised in AAV patients with active disease.
- RAGE expression is increased in PBMC and in AAV biopsy tissue while sRAGE is not.
- Co-stimulation with S100A8/A9 and ANCA enhances pro-inflammatory cytokine production.

Introduction

The ANCA-associated vasculitides (AAV) are a group of systemic autoimmune diseases that encompass distinct clinical phenotypes including granulomatosis with polyangiitis (GPA), where patients typically exhibit anti-PR3 ANCA, microscopic polyangiitis (MPA), where patients often exhibit anti-MPO ANCA, eosinophilic granulomatosis with polyangiitis (EGPA) and renal limited vasculitis [1, 2].

In common, these conditions feature the necrotizing inflammation of small and medium-sized blood vessels,

[1]Department of Immunology and Inflammation, Centre for Inflammatory Disease, Imperial College London, Hammersmith Hospital, London, UK, [2]Nephrology and Dialysis Unit, Ospedale di Circolo e Fondazione Macchi, ASST-Settelaghi, Varese, Italy and [3]Institut D'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain

Submitted 18 May 2020; accepted 4 May 2021

Correspondence to: Theresa H. Page, Centre for Inflammatory Disease, Imperial College London, 9th floor, Commonwealth building, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.
E-mail: Theresa.page@imperial.ac.uk

which frequently results in a pauci-immune, focal, necrotizing, glomerulonephritis (GN), characterized by the presence of infiltrating neutrophils, monocytes and T cells [3–5]. Lung damage (upper or lower respiratory tract granulomatous lesions or alveolar haemorrhage) is also common in AAV so that lung and kidney damage account for much of the morbidity and mortality associated with these conditions. At present, treatment of disease flares and induction therapy in AAV is based on the use of non-specific immunosuppressants [6–9]. Biomarkers that might be useful for accurately predicting flares, monitoring disease progress, or therapeutic efficacy are not yet available, and although ANCA levels are used in clinical practice to monitor AAV activity, there is no clear-cut correlation, especially in the absence of kidney involvement [10]. Thus, there remains a clinical need for reliable biomarkers that might be useful for predicting flares and monitoring disease progress or therapeutic efficacy, and for more targeted treatments.

Danger-associated molecular pattern (DAMP) molecules such as the calgranulins (S100A8/A9 or calprotectin and S100A12) and HMGB1 are expressed at high levels in monocytes and neutrophils, cells critical to the pathogenesis of AAV [11].

Cellular activation results in both active and passive release of these molecules and their expression on the cell surface allowing interaction with two main receptors, TLR4 and the receptor for advanced glycation end products (RAGE) [12, 13]. The literature surrounding the role and relative importance of these two receptors is complex, and although it is generally agreed that both receptors bind calgranulins and HMGB1, the binding affinity, binding sites and cofactor requirements differ for each ligand. Differences in ligand responses (e.g. cytokine production, migration) are seen when binding to the same receptor and both TLR4 and RAGE dependent/independent pathways have been described, with individual ligands preferring one or the other receptor depending on the cell type [14–20].

Levels of TLR4 are upregulated in AAV renal biopsies and our own studies have shown increased CD14 expression in monocytes from PR3 ANCA patients [21–23]. However, RAGE, which has been linked to vascular pathologies including cardiovascular disease (CVD), type 2 diabetes (T2DM), Kawasaki disease and Takayasu's arteritis [24–28] has not been studied in AAV. Signalling via RAGE induces further RAGE expression so that this receptor is considered a significant cause of signal amplification in chronic inflammation [29]. However, soluble forms of RAGE (sRAGE), produced by alternative gene splicing or by cell surface cleavage of the full-length receptor (endogenous soluble RAGE or esRAGE) are also released [30, 31], which sequester RAGE ligands and counteract the effects of both DAMP and RAGE upregulation. The relative levels of RAGE and sRAGE may thus determine the effects of DAMP expression *in vivo*, and studies with small molecule inhibitors or with recombinant sRAGE have proved effective therapies in animal models of CVD, T2DM and Alzheimer's disease [32, 33].

To date, circulating DAMP levels in vasculitis patients have been studied in isolation, revealing raised S100A8/A9 in active adult AAV, and raised S100A12 in active PR3 ANCA and ANCA-negative childhood vasculitides [34–36], while data on levels of HMGB1 have been mixed [37–39]. However, understanding the relationship between different DAMPs in the same patient and their relative value as markers of disease is lacking, as is information on the expression and function of RAGE and sRAGE. Understanding the role of this axis in AAV disease pathology will provide understanding of the value of these molecules both as disease markers and as potential therapeutic targets.

Methods

Patient samples

This study was conducted under local Research Ethics Committee (London-West London) approval; Study Title: Analysis of lymphocyte responses to autoantigen in patients with glomerulonephritis (REC No. 04/Q0406/25) and all patients provided written, informed consent prior to recruitment. We included patients with a diagnosis of systemic vasculitis consistent with the Chapel Hill Consensus Conference definitions [2] and positive ANCA serology or patients with biopsy proven IgA nephropathy (IgAN) or anti-GBM disease. Serum, plasma, PBMC and whole blood samples were collected, aliquoted and stored at -80°C before use. Blood for isolation of PBMC was collected in EDTA-coated BD vacutainer® tubes and processed as soon as possible. Clinical characteristics and laboratory parameters were recorded on the day of sample collection. Disease activity was scored using version 3 of BVAS. Active patients were defined as those with a BVAS score of more than zero and remission as a BVAS score of zero. We excluded patients who were receiving extra-corporeal therapies (haemodialysis, plasma exchange) and those with an eGFR of <15 from serum and plasma measurements, and those with concomitant diabetes, malignancies, or infections from all groups. Patient clinical characteristics are shown in Table 1. Patients in AAV group 1 are those whose sera were analysed for calprotectin and sRAGE. Where appropriate samples were available, a subset of these patients (AAV group 2) were also analysed for S100A12, HMGB1, *AGER* and es*AGER*. Patients were classified as GPA or MPA according to clinical characteristics and ANCA serology was defined as PR3+ or MPO+.

ELISAs

Calprotectin (S100A8/A9) (Biolegend, San Diego, CA, USA), HMGB1 (2B Scientific, Oxford, UK), S100A12 (EN RAGE) (R&D systems, Abingdon, UK) and sRAGE (BioTechne, ABingdon, UK) ELISAs were used according to the manufacturer's instructions. Cross-reactivity between the ELISAs for S100A12 and S100A8/A9 was discounted by the demonstration that the S100A12 ELISA is unable to detect exogenously added recombinant S100A8/A9 (not shown.) Data shown represent mean

TABLE 1 Characteristics of the patients used in this study

GROUP	MPO+/PR3+	M/F	Act	Rem	AGE	K	ENT	L	J	NEURO	GI	TREATED[a]
AAV 1 ($n = 74$)	41% MPO+ ($n = 30$) 59% PR3+ ($n = 44$)	52% M 48% F	44%	56%	58 (24–94)	69%	46%	49%	11%	6.5%	5%	75%
AAV 2 ($n = 37$)	40.5%MPO+ ($n = 15$) 59.5% PR3+ ($n = 22$)	58% M 42% F	43%	57%	59 (27–80)	77%	42%	50%	8%	5%	3%	70%
GBM ($n = 7$)	14% MPO ($n = 1$)	57% M	28%	72%	44 (31–54)	85%	0%	43%	0%	0%	0%	14%
IgAN ($n = 9$)	14% PR3 ($n = 1$) 0%	43% F 45% M 55% F	NA	NA	45 (28–70)	100%	NA	NA	NA	NA	NA	44%

PR3+: PR3 ANCA; MPO+: MPO ANCA; M/F: male/female; Act; active disease; Rem; remission; AGE: mean (min–max), organ involvement shown in each column (K: kidney; ENT: ear, nose, throat; L: lung; J: joint; NEURO: neurological; GI: gastrointestinal tract). [a]treatments included prednisolone, MMF, CYC, AZA, rituximab. NA: not available.

values of duplicate or triplicate samples. ELISAs were read using a BioTeck EL800 plate reader and analysed using Gen5 software.

Tissue samples and immunohistochemistry

Pathological kidney and lung samples were obtained from the Tissue Bank of the Imperial College Healthcare NHS Trust. Normal kidney tissue was collected from organs harvested for transplantation but not used because of inadequate perfusion. Normal lung tissue was from unaffected tissue in samples from resection for lung carcinoma. Formalin fixed, paraffin embedded tissue was stained after antigen retrieval (10 mM sodium citrate pH 6.0, 30 min, 95°C) overnight at 4°C, with a mouse monoclonal antibody specific for the C-terminal V domain of human RAGE (Santa Cruz, CA, USA), or with antibodies for CD3 (Dako, Stokport, UK), CD20 (Abcam, Cambridge, UK), CD68 (Dako, Stockport, UK) or myeloperoxidase (Dako, Stockport, UK).

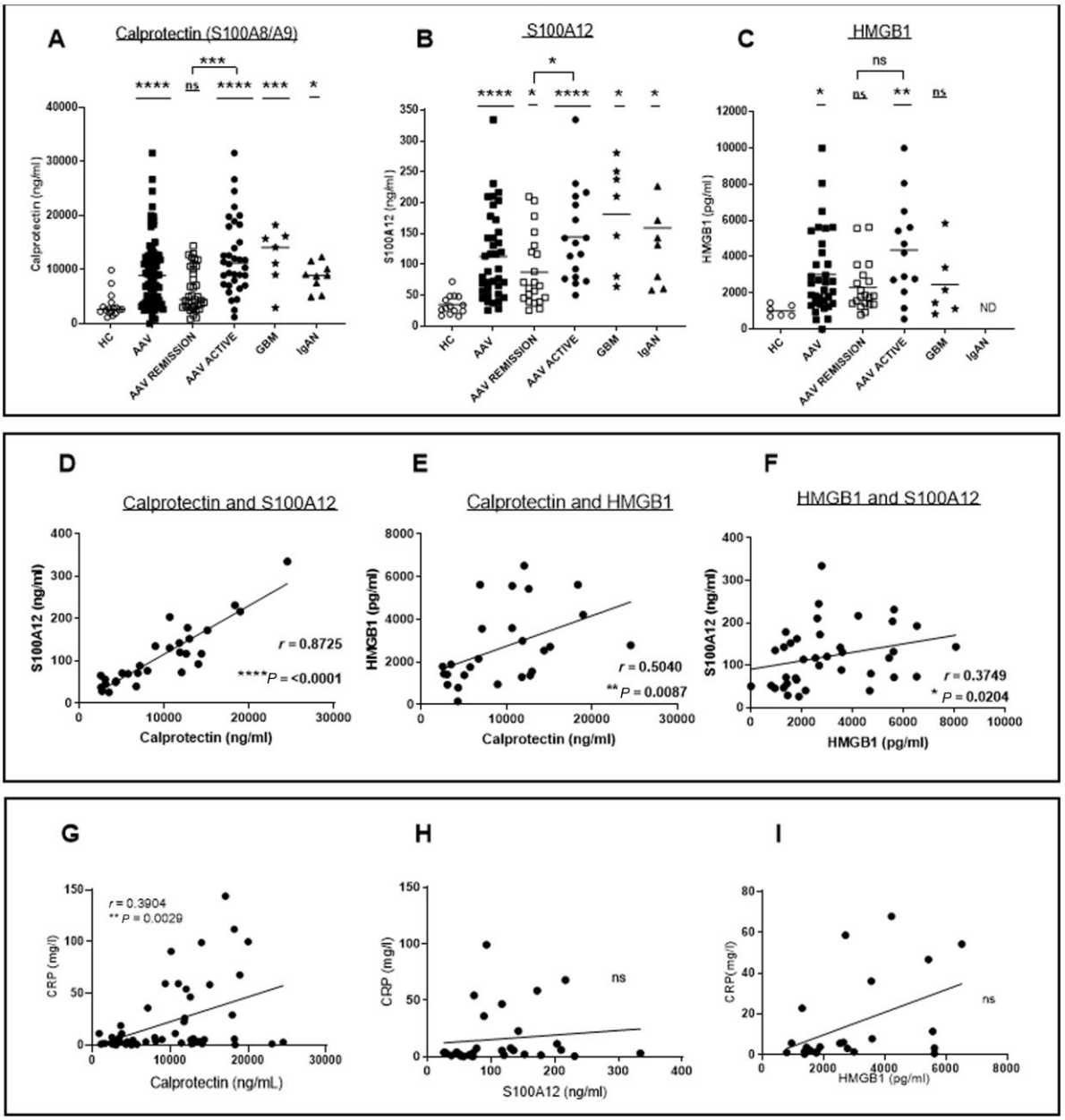
PBMC and ANCA preparation and stimulation

PBMC were isolated from healthy volunteers by density gradient centrifugation over a Histopaque® 1077 gradient. ANCA IgG was prepared from plasma exchange fluid collected during treatment of AAV patients with active disease. IgG was purified by passage of ammonium sulphate precipitated immunoglobulins over a protein G column and stored at −20°C. IgG preparations contained <0.01EU/ml of endotoxin as determined by amoebocyte LAL assay (Thermofisher, Dartford, UK). Cells were resuspended in DMEM/10% FCS and primed for 30 min with 2 ng/ml TNF (Peprotech, London, UK) followed by the addition of ANCA IgG (100 μg/ml) for the indicated times. For co-stimulation experiments, recombinant S100A8/A9 (R + D, Abingdon, UK) was added to a final concentration of 2 μg/ml. FPS-ZM1 (Stratech, Ely, UK) and CLi-098 (InvivoGen, Tolouse, France) were added to a final concentration of 500 nM 1 h before the addition of S100A8/A9. Individual reagents were pretreated with 100 μg/ml Polymixin-B for 2 h at 37°C and then added to cultures to achieve a final concentration of 10 μg/ml. Cells were incubated at 37°C/5% CO_2 in a humidified atmosphere. Supernatants were harvested at the times indicated and stored at −20°C before analysis.

PCR

Total RNA was prepared using a Qiagen RNEasy kit and converted to cDNA using Superscript™ III. PCR reactions were performed with a SyGreen Hi-ROX mix from PCR Bio (London, UK) in an Eppendorf Mastercycler® ep384 machine and 2^-dCt values calculated using GAPDH as a housekeeping gene. All PCR reactions were repeated on at least two separate occasions with similar results. Oligos: total RAGE Forward 5′GAAACTGAACACAGGCCG GA3′, Reverse 5′GAAGGAAGAGGGAGCCGTTG3′, esRAGE Forward 5′ATGAAGGATGGTGTGCCCT3′, Reverse 5′TT TATCAAACCCCTCACCTGC3′.

Fig. 1 Levels of circulating DAMPs in AAV patients and healthy controls



Concentrations of **(A)** Calprotectin, and **(B)** S100A12 in serum and **(C)** HMGB1 in plasma of healthy control (HC) all AAV patients (AAV) (PR3+ and MPO+, see Table 1) in clinical remission (AAV REMISSION) or with active disease (AAV ACTIVE), anti-GBM patients (GBM) and IgAN patients (IgAN). ND= not determined. Correlations between levels of calprotectin and S100A12 **(D),** Calprotectin and HMGB1 **(E)** and S100A12 and HMGB1 **(F)** in individual AAV patients. Correlations between levels of Calprotectin **(G),** S100A12 **(H)** and HMGB1 **(I)** with CRP levels in individual AAV patients. Spearman or Pearson r and *P* values are shown on the graphs. Statistical tests used are shown in Supplementary Table S3, available at *Rheumatology* online. Data shown are mean values of duplicate or triplicate samples. Horizontal lines show mean values. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns: not significant.

Statistics

Statistical significance was calculated using GraphPad Prism 8 software. One-way ANOVA was used for multiple comparison tests with either Kruskal–Wallis, Brown Forsythe Welch or Sidak's tests depending on data distribution. Welches or Mann–Whitney t-tests were used for single comparisons. Details of data distributions, tests, *post hoc* tests and *P*-values are given in Supplementary Table S3, available at *Rheumatology* online. Correlations were calculated using Pearson or

Spearman correlations according to the data distribution ***$P < 0.0001$, **$P = 0.01$, *$P < 0.05$, not significant $P > 0.05$.

Results

Levels of circulating DAMPs are raised in AAV patients with active disease

Sera from AAV patients, collected from the vasculitis clinic at Hammersmith Hospital were screened for levels of the calgranulins, calprotectin (S100A8/A9, calgranulin A/B) and S100A12 (calgranulin C). Plasma was screened for HMGB1. Levels of all three DAMPs were significantly raised in the circulation of active AAV patients (Fig. 1A–C) compared with healthy controls. Patients in remission showed no significant difference in their levels of calprotectin and HMGB1 when compared with healthy controls but did have significantly raised levels of S100A12 (Fig. 1B).

Comparison of the level of DAMPs in individual AAV patients showed significant correlations, particularly between the level of the calprotectin and S100A12 (Fig. 1D–F). Serum CRP levels in AAV showed a similar trend to those of HMGB1 and S100A12, but only reached significance with calprotectin (Fig. 1G–I).

There were no significant differences in the levels of circulating calgranulins or of HMGB1 between male and female patients (both remission and active) (Supplementary Fig. S1A–C, available at *Rheumatology* online) or between those with PR3 ANCA or MPO ANCA (Supplementary Fig. S1D–F, available at *Rheumatology* online). Circulating calgranulin/HMGB1 levels did not differ between AAV patients experiencing kidney disease and those who did not (Supplementary Fig. S1G–I, available at *Rheumatology* online).

Raised circulating levels of both calgranulins and HMGB1 are seen in a wide variety of inflammatory conditions and are not specific for AAV. Accordingly, we also observed raised circulating levels of calgranulins in smaller cohorts of both IgAN and anti-GBM patients (Fig. 1A and B). Levels of HMGB1 in anti-GBM patients showed a trend towards an increase but did not reach significance.

Serum sRAGE protein levels are not raised in AAV

Calprotectin, S100A12 and HMGB1 are recognized at the cell surface by TLR4 and the receptor for advanced glycation end products (RAGE). However, soluble decoy forms of RAGE are also produced (sRAGE) that lack a cytoplasmic domain and act to sequester ligands [30, 31]. An increase in circulating sRAGE has been described in some inflammatory conditions and, by reducing ligand availability, may inhibit DAMP-mediated inflammation. We therefore examined serum levels of sRAGE in AAV.

We found that AAV patients exhibited a wider range of serum sRAGE levels than healthy controls (255–3737 pg/ml compared with 700–1700 pg/ml in healthy controls) and that a significant number of AAV patients had low levels of sRAGE compared with healthy controls. Levels of sRAGE in patients in remission were not significantly different from healthy controls; however, AAV patients with active disease showed a significant reduction in sRAGE levels compared with healthy controls and to patients in remission (Fig. 2A). Indeed, lower levels of sRAGE (below the tenth percentile of healthy controls) were noted in over 50% (53.6%) of active AAV patients (Fig. 2B). Levels of sRAGE in ANCA patients showed an inverse correlation with plasma levels of HMGB1 but not with serum calprotectin or S100A12 (Fig. 2C–E). Serum sRAGE showed no correlation with ANCA serology (PR3 ANCA/MPO ANCA) or age (not shown). In contrast to AAV patients, reduced levels of circulating sRAGE were not found in IgAN or anti-GBM patients despite increased circulating calgranulin levels in both diseases (Fig. 2A).

Transcription of RAGE and esRAGE is enhanced in PBMCs from active AAV patients

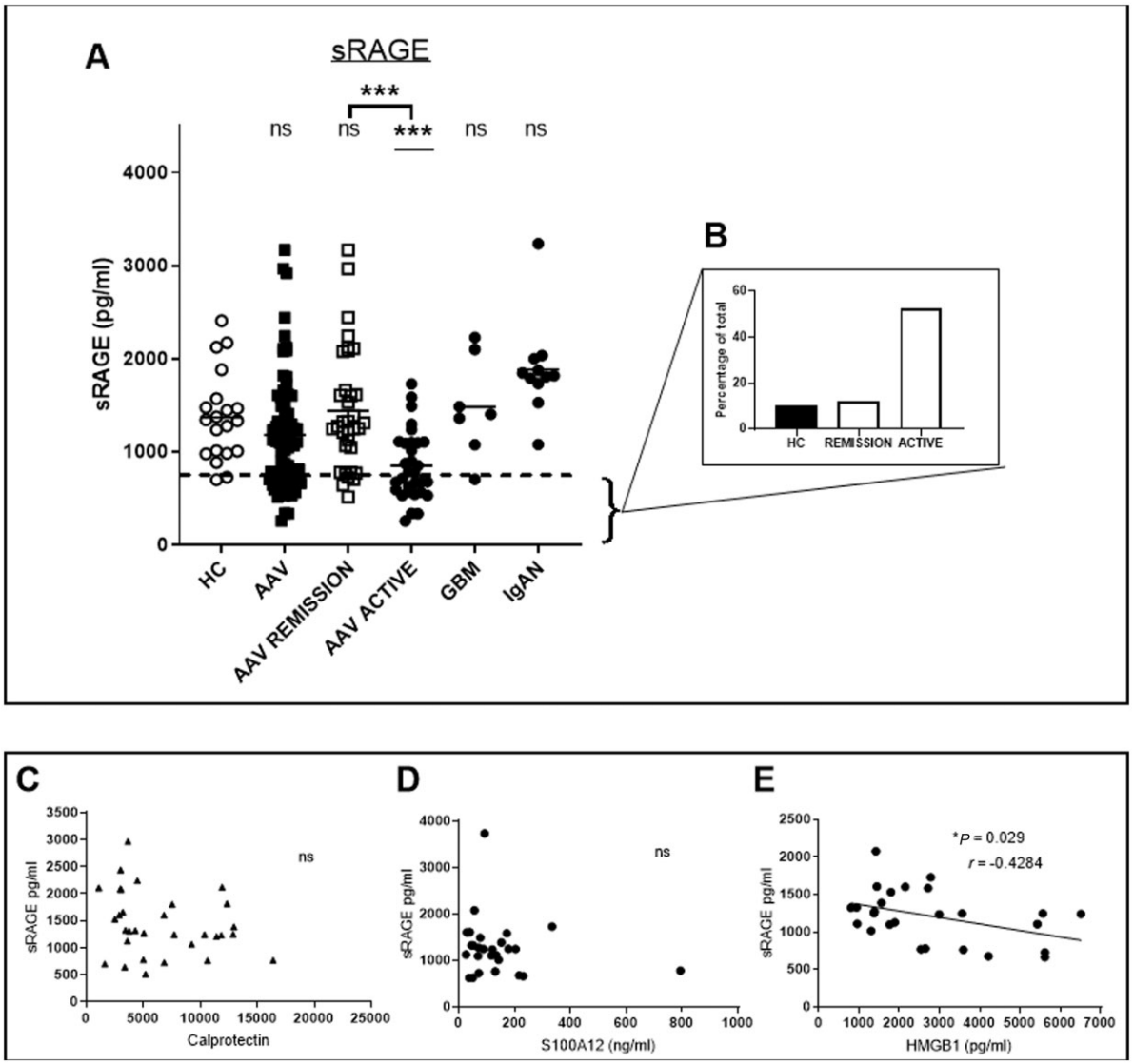
The reduced sRAGE levels in active AAV might occur because of sequestration by the high levels of circulating DAMPs and/or by a reduction in the release of sRAGE. sRAGE is produced either by ADAM-10 mediated cleavage of full-length receptor or by alternative splicing of the RAGE (*AGER*) gene [30, 31]. Reduced transcription of full-length AGER and/or alternatively spliced es*AGER* would therefore lead to reduced circulating sRAGE. To test this hypothesis, we used qPCR to determine the level of transcription of *AGER* and es*AGER* in PBMCs from AAV patients. However, we found that transcription of both total *AGER* (Fig. 3A) and es*AGER* (Fig. 3B) was significantly raised in AAV patients compared with healthy controls, revealing that reduced transcription is unlikely to account for the reduction in sRAGE levels seen. Indeed, transcription of both AGER and es*AGER* was raised in all groups examined, regardless of disease activity.

RAGE is highly expressed on cells infiltrating AAV kidney and lung biopsy tissue

Raised *AGER* transcription in PBMC would be expected to result in raised cell surface expression and hence an increased ability to respond to RAGE ligands. To establish whether cells infiltrating diseased tissue from AAV patients also expressed significant levels of RAGE, we examined the expression of cell-associated RAGE in both AAV kidney ($n = 8$) and lung ($n = 3$) biopsy tissue using an antibody specific for the ligand binding domain of RAGE.

In non-diseased lung tissue, RAGE expression was restricted to scattered alveolar epithelial cells, smooth muscle and vessel endothelia (Fig. 4A and B). In contrast, we found that cells within lung granulomas (GPA patients) stained strongly for RAGE (Fig. 4C). In non-diseased kidney tissue, RAGE expression was either undetectable, or was present at low levels on glomerular and vascular endothelium and smooth muscle (Fig. 4E and F). In contrast, strongly staining infiltrating RAGE+ cells were detected both within diseased glomeruli and

FIG. 2 Circulating levels of sRAGE in AAV patients and healthy controls



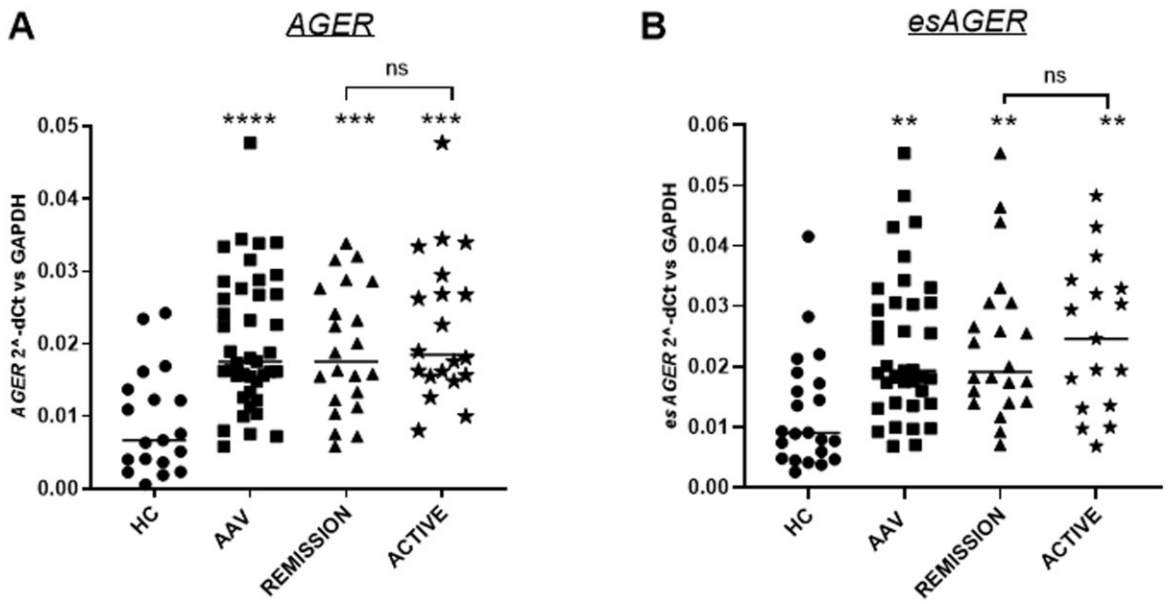
(A) Concentrations of sRAGE in serum of healthy control (HC) and all AAV patients (AAV), AAV patients in clinical remission (AAV REMISSION) and those experiencing active disease (AAV ACTIVE), anti-GBM patients (GBM) and IgAN patients (IgAN). Dotted line indicates tenth percentile of healthy controls. **(B)** percentage of AAV patients showing sRAGE levels below the tenth percentile of HCs. Data shown are mean values of triplicate samples. Horizontal lines show mean values. Statistical tests used are shown in Supplementary Table S3, available at *Rheumatology* online. ***P <0.001, **P <0.01, *P <0.05, ns: not significant. Correlations between circulating sRAGE and **(C)** calprotectin, **(D)** S100A12 and **(E)** HMGB1 in AAV patients. Points represent mean values of duplicate samples from the same individual. Spearman P-value is shown.

within extra-glomerular regions of cellular infiltrate (Fig. 4G). Staining of sequential kidney biopsy sections from an MPO ANCA patient with crescentic disease demonstrated that RAGE co-localized with macrophage (CD68), B cell (CD20), T cell (CD3) and neutrophil (MPO) markers on infiltrating cells (Fig. 4I–M). Similar staining patterns were seen on infiltrating cells regardless of disease (PR3 ANCA or MPO ANCA) or histopathological class examined (mixed and crescentic). We did not observe significant changes in RAGE expression on endothelia (glomerular, peri-tubular or larger vessel) in these biopsies, suggesting that RAGE-mediated responses in these tissues may be dominated by those of infiltrating cells.

ANCA-mediated cytokine production in PBMCs is enhanced by co-stimulation with calprotectin

RAGE-ligand interaction results in enhanced transcription of RAGE so that this receptor is known to amplify

FIG. 3 Transcription of *AGER* and es*AGER* are raised in PBMC from AAV patients



qPCR analysis of *AGER* (**A**) and *esAGER* (**B**) in PBMC from healthy control (HC) and AAV patients including patients in remission and those with active disease. Horizontal lines represent mean values. Statistical tests used are shown in Supplementary Table S3, available at *Rheumatology* online. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

inflammatory responses. Our observation that AAV PBMCs have increased *AGER* mRNA levels therefore suggests that these cells are directly responding to circulating DAMPs. While this may not differentiate AAV patients from several other inflammatory conditions, AAV patients are unique in their expression of ANCA.

We therefore examined cytokine production in PBMCs from healthy donors stimulated with a combination of calprotectin and ANCA to understand if co-stimulation enhanced cytokine production in these cells. PBMC primed with TNF and stimulated with a combination of calprotectin and MPO ANCA produced significantly more IL-8 than cells stimulated with either TNF + ANCA or TNF + calprotectin alone (Fig. 5A and Supplementary Fig. S2, available at *Rheumatology* online). For cells stimulated with PR3 ANCA, where TNF+ANCA alone does not consistently induce cytokine production (Fig. 5B, Supplementary Fig. S2, available at *Rheumatology* online and [40]), the additional presence of calprotectin induced significant IL-8 production in one of the three ANCA donors tested (Fig. 5B). IgG preparations from healthy controls were not able to enhance IL-8 production.

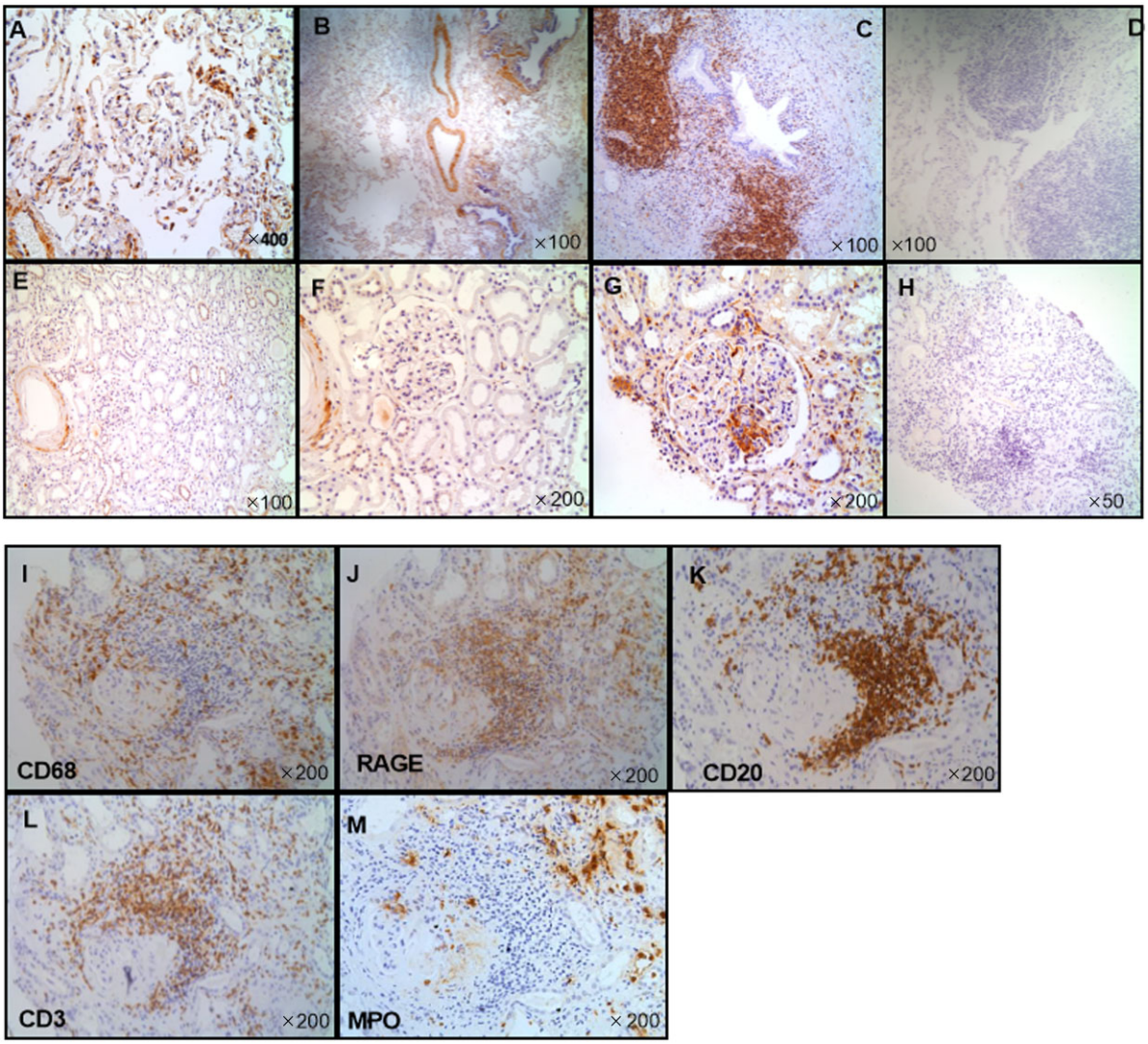
To determine the relative contribution of signalling via TLR4 and/or RAGE, experiments were also performed in the presence of small molecule inhibitors specific for TLR4 (Cli-095/TAK242) and/or RAGE (FPS-ZM1). Our data show that there is a contribution from both receptors with optimal inhibition of IL-8 production being seen in the presence of both inhibitors together (Fig. 5 and Supplementary Fig. S2, available at *Rheumatology* online) in most cases (MPO and PR3 ANCA stimulation) where significant co-stimulation is seen.

Discussion

Here we have shown that AAV patients with active disease have raised circulating levels of calprotectin S100A12 and HMGB1. For patients in remission, levels of calprotectin and HMGB1 are not significantly different from those of healthy controls, while levels of S100A12 remain raised. This observation differs from findings in childhood vasculitides, where S100A12 levels were normalized in remission [36] and may reflect a degree of cellular activation in adult remission patients, a hypothesis that is supported by our observation that RAGE/sRAGE transcription also remains raised in remission. Interestingly, the small group of remission patients who suffered a relapse a short time after sample collection show significantly higher levels of circulating calgranulins than those who stayed in remission (not shown). Raised circulating DAMPs are seen in both PR3 ANCA and MPO ANCA patients and are not restricted to patients experiencing kidney involvement, suggesting that the DAMP: RAGE/TLR4 axis is a common pathogenic pathway in both PR3 ANCA and MPO ANCA AAV irrespective of the organs involved.

This is the first time to our knowledge that circulating levels of three separate DAMPs have been measured within individual AAV patients. What drives DAMP production in AAV is unclear, and we could not find a correlation between DAMP levels and ANCA titres (not shown) suggesting that ANCA may not be a direct driver of DAMP release. Our description of the reduced levels of circulating sRAGE is the first of its kind in AAV patients and reveals that sRAGE levels are unlikely to be

FIG. 4 High levels of RAGE expression in infiltrating cells in AAV lung and renal biopsies



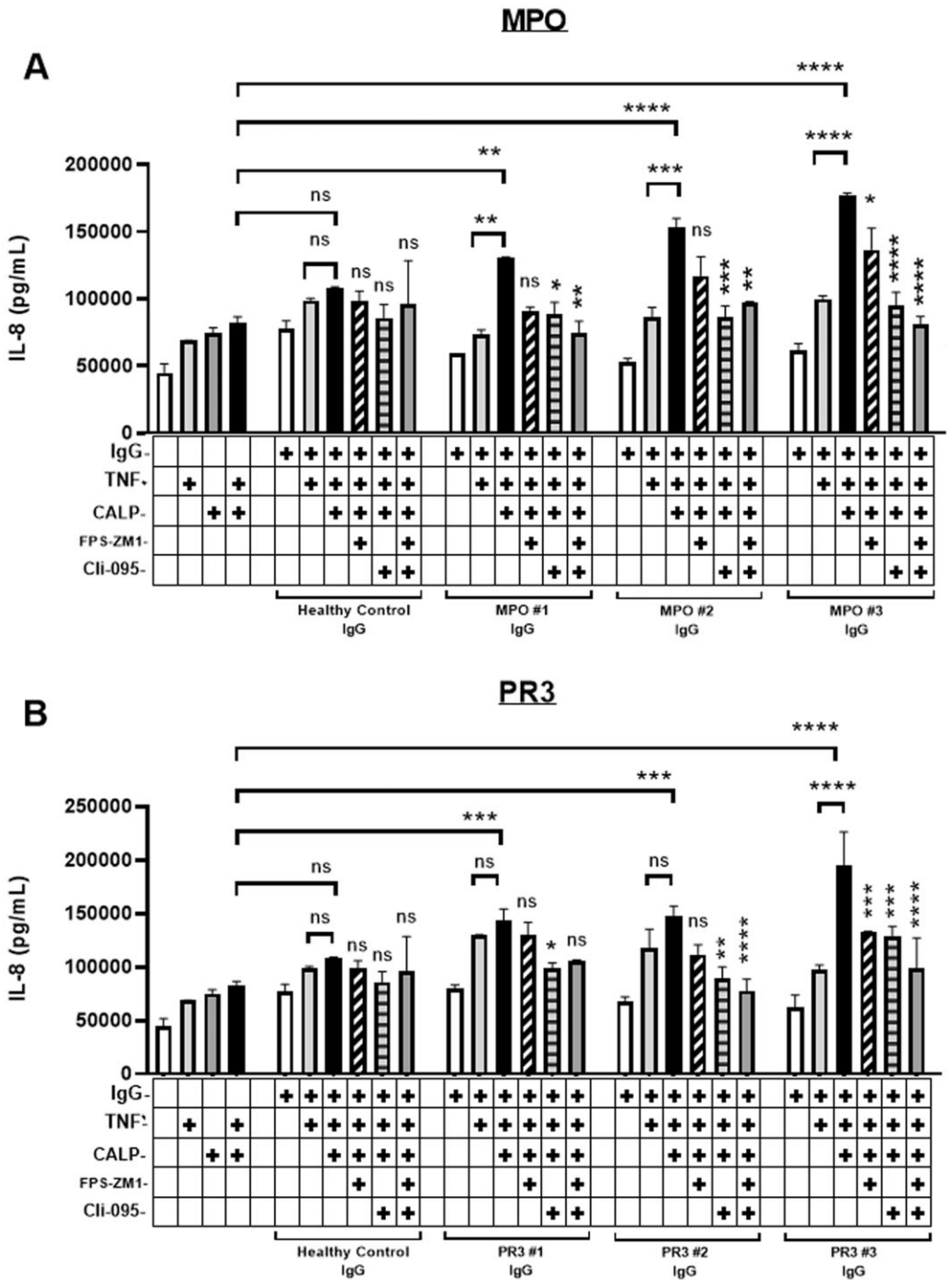
RAGE staining of (**A** and **B**) healthy lung tissue, (**C**) AAV (GPA) lung granuloma, (**E** and **F**) healthy kidney and (**G**) AAV kidney biopsy. Isotype control antibody staining of AAV lung granulomas and renal biopsy tissue are shown in (**D**) and (**H**), respectively. Sequential renal biopsy sections (MPO+, crescentic class) stained for the presence of cells expressing (**I**) CD68, (**J**) RAGE, (**K**) CD20, (**L**) CD3 and (**M**) Myeloperoxidase (MPO). Magnifications are shown on each panel.

sufficient to modulate the response to circulating DAMPs. This was particularly striking in AAV patients with active disease where almost 50% of patients showed sRAGE levels below the tenth percentile levels of healthy controls. In contrast, a similar reduction in sRAGE was not seen in the IgAN and anti-GBM patients examined, and as both anti-GBM and IgAN patients showed comparable increases in calgranulin levels to those of the AAV patients, sRAGE sequestration by ligands appears unlikely to fully account for the reduced sRAGE in active AAV. The reasons for these differences are unclear at present but suggest that there may be a degree of disease-specific regulation of sRAGE levels in AAV that is not shared by other types of GNs.

In line with increased ligand availability in AAV patients, we have shown that transcription of both total *AGER* and es*AGER* is enhanced in PBMC from active AAV patients. As *AGER* transcription is upregulated by ligand binding, this observation suggests that circulating PBMC are encountering and responding to RAGE ligands in AAV patients. Our observation that RAGE transcription is also raised in patients in remission is similar to our previous observation of raised CD14 levels in the monocytes of PR3 ANCA patients in remission [23], adding weight to the argument that remission patients remain in a heightened state of activation.

In addition to raised levels of DAMPs and reduced levels of sRAGE, we show here that RAGE protein is

FIG. 5 Co-stimulation with ANCA and calprotectin enhances IL-8 production in PBMCs



PBMC from a healthy donor were primed with TNF followed by exposure to IgG preparations from healthy controls (HC), MPO ANCA (MPO) (**A**) or PR3 ANCA (PR3) (**B**) from three individual patients (#1, #2, #3) for 4 hours and calprotectin (2μg/ml) for 18hours. FPS-ZM1 and/or Cli-095, where used, were added 1h before calprotectin. Levels of IL-8 in the supernatant were measured by ELISA. The experiment was performed in the presence of polymyxin B. Data shown is a representative example, additional experiments are shown in Supplementary Fig. S2, available at *Rheumatology* online. Data represents mean (+SD) of experimental replicates. Statistical tests used are shown in Supplementary Table S3, available at *Rheumatology* online. Significance of IL-8 levels in cells treated with FPS-ZM1 or Cli-095 is relative to cells with ANCA+T+C. ***P <0.001, **P <0.01, *P <0.05, ns: not significant.

highly expressed on cells (CD68+ cells, T cells, B cells and neutrophils) infiltrating both AAV lung and kidney biopsy tissue. Lung pathology is a significant cause of patient morbidity and mortality in AAV; ~50% of the patients studied here had lung disease and the high level of RAGE expressed on cells within lung granulomas suggests that this molecule may be an important mediator of inflammation and fibrosis in AAV lung tissue. High levels of RAGE on cells infiltrating kidney tissue suggest the same may apply to AAV kidney disease, and reveal a different expression pattern to TLR4, where raised levels are reported on glomerular endothelial cells and podocytes as well as infiltrating immune cells [21, 22]. The relative contributions of both receptors to DAMP-mediated pathology are likely to be complex and may be influenced by both the cell type and the DAMP in question, but it is interesting to note that some murine models of AAV require a TLR stimulus for disease induction [40]. Studies to determine whether each receptor serves a different role in pathology, or if there is a degree of redundancy, are required.

Activation of the DAMP: RAGE axis is not unique to AAV patients and has been described in a variety of inflammatory scenarios. AAV patients, however, are unique in their expression of ANCA, which can stimulate a range of pro-inflammatory responses from monocytes and neutrophils [41, 42]. Potential links between DAMPs and AAV pathogenesis have been highlighted by studies showing enhanced degranulation and respiratory burst in neutrophils treated with ANCA and HMGB1, as well as increases in neutrophil surface expression of PR3 and release of MPO [43]. Our data now adds considerable strength to this argument by showing that co-stimulation with calprotectin and ANCA can boost cytokine production in PBMCs in a mechanism that uses both RAGE and TLR4.

Taken together, we reveal that the raised levels of circulating DAMPs in active AAV, coupled with reduced levels of decoy receptor (sRAGE) provide an environment where cells expressing surface RAGE receive enhanced levels of signalling. In turn, this results in increased RAGE expression in PBMC, and ANCA/calprotectin co-stimulation of PBMC produces enhanced levels of pro-inflammatory cytokines in a TLR4 and RAGE-dependent manner. It is likely, therefore, that the DAMP: RAGE/TLR4 axis provides a platform for the uncontrolled inflammation/fibrosis/vascular endothelial cell pathology that are hallmarks of active disease in AAV. RAGE-specific inhibitors and sRAGE mimetics have already been tested in models of several diseases, including diabetes and Alzheimer's with some success [24, 32, 33]. Targeting this axis may therefore provide a feasible approach for modulating disease severity in AAV.

Acknowledgements

We acknowledge support from the National Institute for Health Research Imperial Biomedical Research Centre. Part of this work was presented in abstract form at the 19th International Vasculitis and ANCA Workshop (Philadelphia, USA, 2019). Study design: T.H.P., C.D.P. Performed experiments: T.H.P., F.B., D.C., J.G., J.B. Sample collection: F.B., F.D., S.P.M., M.P. Data acquisition: T.H.P., F.D., S.P.M. Manuscript preparation: T.H.P., C.D.P.

Funding: This work was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust. T.H.P. was supported by grants from Vasculitis UK (Grant No. 1705) and the NIHR Imperial Biomedical Research Centre (RDA28), F.B. was supported by an ERA-EDTA Young Fellowship (LTF 212–2015), D.C. was funded by the Auchi foundation (Imperial healthcare charity No. 5083), J.B. was funded by a grant from KRUK (Grant No. JF-S/IN/2015/1). F.D. was supported by the NIHR Imperial Biomedical Research Centre (RDA28). M.P. and S.P.M. were in receipt of National Institute for Health Research Academic Clinical Fellowship and clinical Lectureships, respectively.

Disclosure statement: C.D.P. has an advisory role for Cell Medica. S.P.M. has received honoraria from Celltrion and Rigel Pharmaceuticals, and has undertaken consultancy work for GSK. All other authors declare no competing interests.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author. Data are available upon reasonable request by any qualified researchers who engage in rigorous, independent scientific research, and will be provided following review and approval of a research proposal and Statistical Analysis Plan (SAP) and execution of a Data Sharing Agreement (DSA). All data relevant to the study are included in the article.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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