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Immunomodulatory role of proteinase-activated receptor-2

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Abstract

Objective—Proteinase-activated receptor-2 (PAR₂) has been implicated in inflammatory articular pathology. Using the collagen-induced arthritis model (CIA) the authors have explored the capacity of PAR₂ to regulate adaptive immune pathways that could promote autoimmune mediated articular damage.

Methods—Using PAR₂ gene deletion and other approaches to inhibit or prevent PAR₂ activation, the development and progression of CIA were assessed via clinical and histological scores together with *ex vivo* immune analyses.

Results—The progression of CIA, assessed by arthritic score and histological assessment of joint damage, was significantly ($p < 0.0001$) abrogated in PAR₂ deficient mice or in wild-type mice administered either a PAR₂ antagonist (ENMD-1068) or a PAR₂ neutralising antibody (SAM11). Lymph node derived cell suspensions from PAR₂ deficient mice were found to produce significantly less interleukin (IL)-17 and IFN γ in *ex vivo* recall collagen stimulation assays compared with wild-type littermates. In addition, substantial inhibition of TNF α , IL-6, IL-1 β and IL-12 along with GM-CSF and MIP-1 α was observed. However, spleen and lymph node histology did not differ between groups nor was any difference detected in draining lymph node cell subsets. Anticollagen antibody titres were significantly lower in PAR₂ deficient mice.

Conclusion—These data support an important role for PAR₂ in the pathogenesis of CIA and suggest an immunomodulatory role for this receptor in an adaptive model of inflammatory

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arthritis. PAR₂ antagonism may offer future potential for the management of inflammatory arthritides in which a proteinase rich environment prevails.

INTRODUCTION

Rheumatoid arthritis (RA) is a progressive inflammatory arthropathy associated with substantial vascular comorbidity and thereby increased mortality.¹ Therapeutic interventions via aggressive use of conventional and biologic disease modifying agents have significantly improved outcomes but unmet clinical need remains manifest in low remission rates and significant partial or non-responder subpopulations.²³ A key learning point from such studies has been the pivotal role played by cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-6 in disease pathogenesis. It is therefore critical to elucidate those upstream factors that regulate cytokine production. Increasingly, elements of both innate and adaptive immunity are implicated in RA pathogenesis; for example, genome-wide scans implicate genes regulating T cell and B cell activation and novel therapeutics targeting co-stimulation and B cells suppress disease. Signal pathways that modulate cytokine production are also implicated. Collagen-induced arthritis (CIA) comprises a polyarthritis in genetically susceptible mice induced by immunisation with type II collagen, leading in turn to autoreactivity to autologous collagen with consequent downstream responses that include elaboration of effector cytokines,⁴⁻⁶ making this a suitable surrogate of RA for investigation of pathogenic mechanisms.

Proteinase-activated receptor-2 (PAR₂) is one member of a recently discovered family of four cell surface G-protein coupled receptors which has significant roles in inflammation and the coagulation cascade.⁷⁸ Previous studies have employed PAR₂ deficient mice to implicate this receptor in articular inflammation.⁹⁻¹¹ These findings translate to RA in humans since the selective PAR₂ antagonist, ENMD-1068, significantly reduced basal release of TNF α and IL-1 β from RA synovial tissues *ex vivo*.¹² PAR₂ is therefore identified as a novel upstream upregulator of pro-inflammatory cytokines, adding to the growing evidence implicating this receptor as a regulator of innate immune responses.¹³ It is less clear whether PAR₂ regulates adaptive immune responses directly. PAR₂ is expressed on dendritic cells (DCs),¹⁴ which could influence T cell dependent responses via cytokine regulation. We here report the impact of PAR₂ deficiency or blockade upon modulation of arthritis induced by a predominantly adaptive immune challenge, namely CIA.

METHODS

Animals

Adult male DBA/1 mice (body weight ~25 g) were obtained from Harlan (Loughborough, UK), housed in standard plastic cages with food (rodent chow 5001) and water available *ad libitum*, and maintained in a thermoneutral environment (21 $\pm$ 2°C) with 12-h light/dark cycles. PAR₂ deficient mice (PAR₂^{-/-}) and wild-type littermates (PAR₂^{+/+}) on the C57Bl/6J background were generated as previously described.⁹ All procedures were performed in accordance with current UK (Home Office) regulations.

Induction of CIA

In view of the known relative resistance to CIA of C57Bl/6J mice compared with the DBA/1 strain, different protocols were employed for each strain. In DBA/1 mice (8–10 weeks old), lyophilised bovine type II collagen (MD Biosciences, Zurich, Switzerland) was dissolved at 2 mg/ml in 0.05 M acetic acid by gently stirring overnight at 4°C. Immediately prior to induction injections (day 0) the collagen solution and Freund's complete adjuvant (FCA), containing 2 mg/ml heat killed *Mycobacterium tuberculosis* (H37ra, BD Diagnostics, Oxford, UK), were used to prepare a 1:1 emulsion. The emulsion is prepared by adding collagen to FCA dropwise while mixing at low speed in an ice water bath. Solutions and emulsion were kept chilled at all times during mixing and prior to injections. These mice received 2×50 µl intradermal injections of the collagen:FCA emulsion at the base of the tail, that is, 100 µg collagen per animal. Booster injections of 100 µl collagen solution made up 1:1 with 2 mg/ml collagen (in acetic acid) and 0.9% NaCl were given intraperitoneally at day 21. In all, 80% incidence was achieved by day 30 with >80% of animals showing evidence of disease by day 35.

For mice on the C57Bl/6J background a modification of the protocol described by Inglis *et al*¹⁵ was used. Arthritis was induced at 14 weeks. Briefly, 1 mg/ml type II chicken collagen (MD Biosciences, Zurich, Switzerland) in FCA containing 2 mg/ml heat killed *Mycobacterium tuberculosis* as described above. Mice received 4×50 µl intradermal injections of the collagen:FCA emulsion at the base of tail, that is, 200 µg chicken type II collagen per animal. At day 21 postimmunisation, mice received a 100 µl intraperitoneal injection of 200 µg chicken collagen prepared in 0.9% NaCl. Mice were then checked daily to assess clinical scores.

Direct assessment of the inflammatory response was by means of an arthritis index,¹⁶ modified by extending the scale to a maximum of four per paw (supplementary table 1). Animals were terminated after treatment and the draining lymph nodes (LNs) and paws harvested for analysis. As arthritis incidence in the C57Bl/6J strain can be as low as 50%,¹⁵ only mice showing disease activity (defined as an arthritic index ≥ 1) were included in the analyses. To maintain consistency, this rule was also applied to the DBA/1 strain.

Histological analysis

Histological preparation involved harvesting paws, fixing o/n in 10% neutral buffered formalin followed by decalcification in 14% EDTA (pH8) at room temperature, with the EDTA solution changed every 2/3 days for a period of 14 days. Joints were embedded in paraffin wax and frontal sections (6 µm) cut followed by staining with H&E (Sigma-Aldrich, Dorset, UK). Images were digitally captured using a Carl Zeiss AxioCamERc5s camera and an AXIO Lab.A1 microscope.

For each animal, six high power fields were examined and scored by two observers blinded to genotype or treatment. The severity of arthritic changes, in terms of inflammatory cell infiltrate and cartilage damage, was scored on a 0–3 scale for each hind paw and a total score calculated. The scores from the two observers were in close agreement (intraclass correlation coefficient 0.9, 95% CI 0.95 to 0.80) and a mean of their scores is presented.

LN culture

LN cells were collected 5 days after arthritis onset (defined as an arthritic index ≥ 1) from PAR₂^{-/-} mice and wild-type littermates and a single cell suspension prepared. Cells were cultured at 2×10^6 /ml in RPMI-1640 supplemented with penicillin/streptomycin, glutamine, 50 μ M β -mercaptoethanol and 10% foetal bovine serum, in the presence or absence of 50 μ g/ml heat denatured chicken type II collagen (65°C for 30 min), in a final volume of 200 μ l. Incubation of LN cells with plate bound α CD3 (2 μ g/ml) and soluble α CD28 (5 μ g/ml; both from eBioscience, Hatfield, UK) were included as positive controls. After 48 h culture, supernatants were collected and stored at -20°C until they could be analysed for cytokines and chemokines.

In addition, LN single cell suspensions from PAR₂^{-/-} mice and wild-type littermates were also stained for CD11c, F4/80, CD4, CD8 and CD19 populations using commercially available fluorochrome labelled antibodies (eBioscience, UK). Briefly, cells were incubated in fluorescence activated cell sorter (FACS) buffer (PBS/0.1% foetal bovine serum) and Fc block (antimouse CD16/32; eBioscience, UK) at 4°C for 10 min, prior to the addition of antibodies. The incubation was continued at 4°C for a further 30 min. Cells were washed in FACS buffer by centrifugation (150 g for 5 min at 4°C) prior to being resuspended in FACS buffer and 1% paraformaldehyde solution. Samples were then analysed using a BD FACS Calibur and Flowjo 7.2.5 (TreeStar, Oregon, USA).

Cytokine and chemokine analysis

Supernatants from LN cultures were initially assayed for TNF α by ELISA (Invitrogen, Paisley, UK). All other analytes were assayed using a mouse 20-plex kit (Invitrogen, UK) as per manufacturer's instructions and plates were read on a Luminex 100 system.

Analysis of serum anticollagen antibody titres

Serum was collected from responding mice (5 days post-disease onset) and used to analyse collagen specific IgG₁ and IgG_{2a} antibody titres, as outlined in supplementary methods.

Agents

ENMD-1068 (N1-3-methylbutyryl-N4-6-aminohexanoyl-piperazine) is a small molecule PAR₂ antagonist which we have previously shown can inhibit carrageenan-induced joint inflammation.¹⁰ Although lacking potency, ENMD-1068 is selective even at high concentrations as it inhibits PAR₂ mediated vasodilatation but has no effect on vasodilatation induced by either PAR₁ or PAR₄. It also has no effect on acetylcholine mediated vasodilatation and, unlike some PAR₂ antagonists, has no partial agonist activity (see supplementary figure 1). We previously showed that the monoclonal antibody SAM11 (sc13504, Santa Cruz Biotech, California, USA), directed to the sequence SLIGKVDGTSHVTG on the N terminus of the receptor, can also be used to block PAR₂ mediated acute joint inflammation.¹¹

Treatments

Postdisease onset (arthritis score = 1) mice were treated daily by subcutaneous injection of 4 or 16 mg ENMD-1068 in 100 µl 0.9% saline at neutral pH (neutralised with 1M NaOH), or 0.9% saline vehicle alone, for 7 days and the progression of arthritis was monitored daily. In a separate group of DBA/1 mice, SAM11 or IgG_{2a} control antibody was administered intraperitoneally, with a 10 µg loading dose on day 1 of response followed by 1 µg daily dose given thereafter.

Statistics

Data were analysed using Sigmaplot 2.03 (SPSS, California, USA) and are expressed as mean±SEM. Comparisons were performed using the two-tailed Student t test or one- or two-way analysis of variance (ANOVA) as appropriate, post hoc testing being performed using the Bonferroni correction. Where data were non-normally distributed, log₁₀ transformation was performed prior to parametric analysis.

RESULTS

PAR₂ deficiency is protective in murine CIA

C57Bl/6J wild-type mice developed arthritis with an incidence of approximately 60% by day 35 postinduction and exhibited a progressive increase in the arthritis index (figure 1A), commensurate with prior reports using this model strain.¹⁵ In contrast, although PAR₂^{-/-} mice developed arthritis with similar incidence to wild type controls, the severity of disease was substantially reduced in comparison when judged by clinical assessment (arthritis index; p<0.0001, two-way ANOVA; n=10–12). Histological analysis of the paws revealed that wild-type mice showed cartilage erosion and inflammatory cellular infiltration, but appearances in PAR₂^{-/-} mice were similar to naïve mice (figure 1B), and for both of these parameters scored significantly (p<0.0001, t test; n=6–7) lower for severity than the wild-type (figure 1C).

Histological examination of lymphoid organs revealed no significant structural or organisational differences between wild-type or PAR₂^{-/-} mice (supplementary figure 2), and FACS analysis of LN cell subsets showed similar population distributions of CD11c, F4/80, CD4, CD8 and CD19 in both groups of mice (figure 2A). However, examination of IgG₁ and IgG_{2a} antibodies revealed that PAR₂^{-/-} mice had modestly, but significantly, lower titres than wild-type (figure 2B).

Analysis of cytokines and chemokines in supernatants from cultured LN cells suspensions incubated with collagen showed significant differences in the recall response between wild-type and PAR₂^{-/-} mice, particularly striking in respect of IFNγ and IL-17, these being substantially lower in the latter mice (figure 3). Interestingly, significant differences in TNFα, IL-1β, IL-6 and IL-12p40/70 along with MIP-1α and GM-CSF were also observed in these cultures (figure 3). These differences could not be explained by changes in the LN cellular composition exhibited in PAR₂^{-/-} and wild-type mice (figure 2B), suggesting that the differences in cytokine production reflected a functional rather than quantitative phenomenon. Similarly, responses to antiCD3/antiCD28 did not differ significantly between

wild-type and PAR₂^{-/-} mice, indicating no alteration in cell viability and capacity to function after in vitro culture. LN cells from wild-type and PAR₂^{-/-} mice did not secrete detectable levels of IL-1 α , IL-2, IL-4, IL-5, IL-10, IL-13, VEGF, MCP-1, MIG, IP-10, KC and FGF in response to stimulation with collagen in vitro. They did, however, make detectable levels of IL-2, IL-5 and IL-13 in cultures stimulated with α CD3/ α CD28 although these did not differ significantly between groups (data not shown).

Targeting PAR₂ is an effective therapeutic intervention in murine CIA

Having established that PAR₂ could play an immune regulatory role in an adaptive model of arthritis, subsequent studies were restricted to establishing proof of concept for this receptor as a novel therapeutic target in this model. Induction of CIA in DBA/1 mice (performed to allow assessment of exogenous PAR₂ inhibitors) was associated with development of signs of arthritis that were progressive in animals treated with vehicle for up to 7 days. In contrast, animals treated with 16 mg of ENMD-1068 did not show disease progression manifest in the clinical score. Recipients of 4 mg of this compound exhibited an intermediate phenotype suggesting dose responsive inhibition (figure 4A). Both the 4 and 16 mg doses differed from vehicle ($p < 0.00001$ for both, Bonferroni correction) but did not differ from each other. Histopathological analysis demonstrated that in a subset of mice treated with 16 mg ENMD-1068, there was significantly less cellular infiltration ($p < 0.005$, t test) and cartilage damage ($p < 0.0002$, t test) compared with vehicle treatment (figure 4B). Inflammatory hyperaemia of the paw, assessed by laser Doppler imaging, was also attenuated in mice treated with ENMD-1068 (supplementary figure 3).

Essentially, similar results were obtained in this strain of mice using the PAR₂ antibody SAM11, namely significant ($p = 0.017$, 2-way ANOVA, $n = 6$) reduction in arthritic (figure 5A) and histological damage (figure 5B) scores compared with isotype IgG_{2a} control antibody.

DISCUSSION

This is the first study to demonstrate that either genetic deletion of PAR₂ or therapeutic intervention can substantially inhibit collagen-induced murine arthritis. We previously found that disease severity in an innate immune driven model of arthritis (FCA monarthritis) was substantially abrogated in PAR₂^{-/-} mice.⁹ The current study advances our earlier work by identifying an important role for PAR₂ in a more clinically relevant model of arthritis, namely one characterised by having an adaptive immune response.

PAR₂^{-/-} mice had reduced arthritic and histological damage scores compared with wild-type littermates. The lower incidence and magnitude of response to CIA in the C57Bl/6J strain is widely recognised, and while severity and progression were reduced in PAR₂^{-/-} mice compared with wild-type, no difference in incidence was noted between groups in the present study. Importantly, substantial reduction in antigen recall induced cytokine and chemokine production by LN cultures, particularly IFN γ and IL-17, was observed. This is consistent with PAR₂ modulating T helper cell (Th)1 and Th17 responses, thereby influencing immunological response to antigen challenge. Whether this is a direct or indirect effect of PAR₂ on the adaptive immune response remains unclear and is not tested in the

current experimental model. LN from PAR₂^{-/-} mice stimulated with collagen secreted reduced levels of IL-1 β and IL-6, cytokines known to be important in Th17 polarisation of cells,^{17,18} while a reduction in the Th1 polarising cytokine, IL-12,¹⁹ was also observed. If in vivo polarisation is altered in PAR₂^{-/-} mice, this would provide one explanation for the reduced IFN γ and IL-17 observed in the cultures. It is however of note that both human and mouse T cells are capable of expressing PAR₂²⁰ and, moreover, cytokine regulation via PAR₂ signalling has been demonstrated.^{20,21} It is therefore also possible that PAR₂ expressed on T cells may have a direct influence on T cell phenotype and function in vivo which would consequently impact on IL-17 and IFN γ production. While this hypothesis requires additional investigation, we would suggest that our data demonstrate a role for PAR₂ in immune regulation in an adaptive model of arthritis, although the precise mechanism is unclear at present. Potentially this could involve modulation of DC function as DCs derived from the bone marrow of PAR₂ deficient mice release significantly less IL-6, IL-23 and TNF α compared with cells from wild-type mice on exposure to serine protease.²² Furthermore, a PAR₂ activating peptide significantly increased the frequency of mature CD11c^{high} DCs in draining LNs and led to these cells showing increased expression of major histocompatibility complex class II and CD86 in wild-type but not PAR₂^{-/-} mice.²³ However, these authors observed no significant difference in the proportion of CD11c cells in LNs of naïve wild-type and PAR₂^{-/-} mice. Similarly, we observed no change in the CD11c population from LNs of wild-type and PAR₂^{-/-} mice following induction and development of CIA. However, while the DC numbers may not appear altered, their maturation status and cytokine profile may differ and as such this represents an area for future investigation.

Having established proof of concept in the PAR₂^{-/-} mouse studies, we then investigated the potential of therapeutic intervention by targeting PAR₂. Therapeutic administration of the PAR₂ antagonist (ENMD-1068) potently inhibited progression of CIA compared with vehicle-treated mice. The arthritis index was dose-dependently reduced in animals treated with ENMD-1068. Histological analysis of peripheral joints corroborated these findings, both cellular infiltrate and joint damage being substantially lower in treated mice. Parallel findings were obtained in wild-type mice administered the PAR₂ monoclonal antibody SAM11 which showed reduced disease activity compared with mice given isotype control antibody. As a pilot study, inflammatory hyperaemia, assessed non-invasively using laser Doppler imaging, was also reduced in animals treated with ENMD-1068. Taken together, this study argues for an immunomodulatory role for PAR₂ in the pathogenesis of an adaptive immune model of arthritis, even when inhibition is delayed until after the onset of disease. The present report supports our previous observations⁹ and suggests a role for PAR₂ in both innate and adaptive immune-driven arthritic disease processes.

CIA is widely recognised as the 'gold standard' experimental model for investigation of RA and adaptive immune responses. Our findings that both PAR₂ deficiency and therapeutic inhibition ameliorate disease demonstrate a pathogenic role for PAR₂ in inflammatory joint disease. This is supported by the finding of Busso and colleagues¹¹ who reported that PAR₂ deficiency reduced disease activity in antigen-induced monarthritis. Although they were unable to confirm our earlier finding that FCA monarthritis was substantially reduced in PAR₂ deficient mice,⁹ this is likely related to differences in induction protocol on which the

magnitude of chronic inflammatory response is critically dependent. An effective chronic inflammatory response is ensured by a combination of intra-articular and peri-articular injection of FCA.²⁴²⁵ The simpler protocol used by Busso *et al*¹¹ involved using a single intra-articular injection which produces a much smaller inflammatory response.¹¹²⁴ This, coupled with the residual inflammatory response we observed in PAR₂ knock-out mice,⁹ would have made it difficult to distinguish between wild-type and PAR₂ deficient mice in the Busso study.¹¹ This likely explains the difference in result and conclusion between these two studies.⁹¹¹

It is possible that, acting as a proteinase sensor, PAR₂ may form part of an 'alarmin' system operating via innate mechanisms to drive subsequent adaptive responses. Consistent with this is the growing recognition that PAR₂ has important roles in both innate and adaptive immunity (for review see ¹³). The nature of the endogenous proteinases released in LN cultures and which could activate PAR₂ is unknown at present. However, it has been shown that DCs show a variety of proteinase activities including cathepsins and trypsin-like proteinases.²⁶ Macrophages in the LN are known to express a serine proteinase with tryptase-like homology.²⁷ Similarly, B cells are known to show trypsin-like serine proteinase activity.²⁸

PAR₂ has relevance to human disease as it is found in CD68 cells within the synovium of RA patients¹¹¹² and its synovial expression is strongly correlated with inflammatory indices.²⁹ Furthermore, surface expression of PAR₂ is upregulated on CD3 and CD14 cells in RA patients compared with healthy individuals.³⁰ While these observations alone cannot determine the role of PAR₂ in the pathogenesis of RA, a pro-inflammatory role is indicated by our observation that ENMD-1068 potently inhibited TNF α and IL-1 β release from rheumatoid synovial membrane *ex vivo*.¹²

Orally active small molecular weight compounds obviate many of the difficulties of administration associated with current biologic agents, and there is increasing interest in developing such compounds with anti-inflammatory activity, particularly those that might act upstream of existing biologic validated targets. ENMD-1068 shows considerable potential in terms of molecular size and anti-inflammatory action and retains PAR₂ selectivity even at high concentrations (see supplementary figure 1). ENMD-1068 therefore provides a powerful proof of concept for therapeutic targeting of PAR₂. That said, this agent has low potency precluding useful therapeutic development. However, newer PAR₂ antagonists have recently been developed,³¹³² and although potency is still suboptimal, future leads may yield compounds suitable for clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Arthritis index comparing wild-type (n=12) and PAR₂^{-/-} (n=10) mice following development of CIA. The groups differ significantly by two-way ANOVA (see Results section) and post hoc analysis (Bonferroni) showed differences from day 2 after disease onset (*p<0.05; **p<0.02). (B) Histological appearance of joints from the paws of naïve mice (top panel) compared with wild-type (middle panel) and PAR₂^{-/-} mice (bottom panel) stained with H&E. Asterisk denotes cellular infiltrate and arrows indicate cartilage erosion. Scale bar=100 μm]. (C) Histopathological scores comparing PAR₂ deficient (n=6) and wild-type (n=7) mice. ***p<0.0001.

Figure 2.

(A) FACS analysis of draining LN cell subsets, showing no significance differences between groups (n=7–8) 5 days following development of arthritis. (B) Anticollagen antibody titres were significantly lower in $\text{PAR}_2^{-/-}$ compared with wild-type mice (* $p < 0.05$; ** $p < 0.02$).

Figure 3.

Panels indicate concentrations of cytokines and chemokines found in supernatants from lymph node cultures derived from $PAR_2^{-/-}$ (white bars, n=8) and wild-type (black bars, n=8) mice 5 days after disease onset, as indicated by an arthritis score ≥ 1 . *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 4.

(A) Articular index in wild-type DBA/1 mice administered saline vehicle (n=11), or ENMD-1068 at 4 mg (n=5) and 16 mg (n=8) by daily subcutaneous injection after disease onset (arrow). The arthritis score is reduced by treatment at both doses (see two-way ANOVA in Results section). Post hoc analysis showed differences from day 4 after disease onset (* $p < 0.05$; ** $p < 0.01$). (B) Histopathological scores differed significantly between mice treated with vehicle and 16 mg ENMD-1068 for both inflammatory cell infiltration and cartilage damage. *** $p < 0.0001$; ** $p < 0.01$, t test, n=4–8.

Figure 5.

(A) Compared with wild-type treated with control antibody (IgG2a), DBA/1 mice given daily intraperitoneal injection of the PAR₂ neutralising monoclonal antibody SAM11 after disease onset (arrow) show reduced arthritis index, post hoc analysis showing significant differences by day 6 (* $p < 0.05$; ** $p < 0.01$). (B) Scores for both inflammatory cell infiltration and cartilage damage were lower in SAM11 treated mice compared with control IgG2a antibody. * $p < 0.05$, t test, $n = 6$ in each group.