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Ann Rheum Dis. Author manuscript; available in PMC 2012 October 09.

Published in final edited form as:
Ann Rheum Dis. 2012 November ; 71(11): 1872–1880. doi:10.1136/annrheumdis-2011-201180.

The impact of endogenous Annexin A1 on glucocorticoid control of inflammatory arthritis

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Abstract

Objectives—Establish role and effects of glucocorticoids and the endogenous Annexin A1 (AnxA1) pathway in inflammatory arthritis.

Methods—Ankle joint mRNA and protein expression of AnxA1 and its receptors were analysed in naïve and arthritic mice, by real time PCR and immunohistochemistry. Inflammatory arthritis was induced with the K/BxN arthritogenic serum in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice; in some experiments, animals were treated with dexamethasone (Dex) or with human recombinant AnxA1 or a protease-resistant mutant (termed SuperAnxA1). Readouts were arthritic score, disease incidence, paw oedema and histopathology, together with proinflammatory gene expression.

Results—All elements of the AnxA1 pathway could be detected in naïve joints, with augmentation during on-going disease, due to the infiltration of immune cells. No difference in arthritis intensity of profile could be observed between AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice. Treatment of mice with Dex (10 μg i.p. daily from day 2) afforded potent anti-arthritic effects highly attenuated in the knockouts: macroscopic changes were mirrored by histopathological findings and proinflammatory gene (e.g. *Nos2*) expression. Presence of proteinase 3 mRNA in the arthritic joints led us to test AnxA1 and the mutant SuperAnxA1 (1 μg i.p. daily in both cases; from day 2), with the latter one being able to accelerate the resolving phase of the disease.

Conclusion—AnxA1 is an endogenous determinant for the therapeutic efficacy of Dex in inflammatory arthritis. Such an effect can be partially mimicked by application of SuperAnxA1 which may represent the starting point for novel anti-arthritic therapeutic strategies.

Keywords

K/BxN arthritis; Glucocorticoids; Dexamethasone; Neutrophils; Resolution

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Competing interests The authors have no conflicting financial interests.

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INTRODUCTION

There is genuine interest in detailing cellular and molecular events regulating the phase of the resolution of inflammation, with the characterisation of specific players and targets.[1] This interest stems from the appreciation that, in ideal settings, a strong pro-inflammatory response, orchestrated by cytokines, adhesion molecules and chemoattractants[2] must be followed by an anti-inflammatory and pro-resolving phase, which assures spatial and timely control of the response the host organises upon encounter with an insult,[3] leading to resolution and regain of tissue functions.

Annexin A1 is an effector of resolution.[4] Highly expressed in immune cells (e.g. polymorphonuclear cells [PMN] and macrophages), this protein is externalised to exert paracrine and juxtacrine effects, the vast majority of which are mediated by the formyl-peptide receptor type 2, (FPR2/ALX or Fpr2, in rodents).[5] Intriguingly, FPR2/ALX is also the lipoxin A_4 receptor[6] indicating the existence of important – yet not fully appreciated - networks in resolution.[7] Another receptor also advocated to mediate the effects of AnxA1, the formyl-peptide receptor type 1 or FPR1 (Fpr1 in rodents),[8] though it is unclear if and how this receptor binds the full-length protein[9] or whether - more selectively – it would mediate effects of the AnxA1 peptidomimetic peptide Ac2-26.[10]

The majority of experimental approaches used to define properties of proresolving mediators have been reliant on models of acute inflammation, characterised by rapid PMN influx followed by inflammatory monocytes which differentiate into tissue macrophages[3, 11] to phagocytose debris and apoptotic cells and favour tissue repair, a conclusive act in resolution that may require re-epitheliation[12] or a shift in fibroblast phenotype[13]. In this context, the proresolving nature of AnxA1 has been demonstrated in a variety of experimental settings, noting induction of apoptosis, promotion of efferocytosis and tissue repair, spanning from models of acute inflammation (e.g. see[14, 15]) to a model of colitis where delayed resolution was observed in AnxA1$^{-/-}$ mice.[16] An important aspect of AnxA1 biology is its second messenger role in the actions of glucocorticoids, a function mainly addressed in cellular settings [e.g.[17]] or in models of acute inflammation.[18, 19] However, Morand's group applied antigen-induced arthritis to observe reduction afforded by Dex on synovitis, soft tissue inflammation and cartilage erosion was attenuated in AnxA1$^{-/-}$ mice.[20]

The K/BxN serum induced model of inflammatory arthritis is ideal to investigate the impact of specific proresolving pathways. Using this model of active phase of rheumatoid arthritis, Kronke *et al* reported a higher degree of joint inflammation in 12/15-lipoxygenase$^{-/-}$ mice, associated to their inability to synthesise lipoxin A_4.[21] Similarly, animals nullified for proresolving melanocortin receptor type 3 displayed higher degree of arthritis and delayed resolution, accompanied by augmented cytokine expression and osteoclast responsiveness.[22] An equally interesting study investigated the effect of absence of glucocorticoid signal in osteoblasts, by means of restricted transgenic expression of 11β-hydroxysteroid dehydrogenase type 2, in this model of inflammatory arthritis. The outcome was that of a more attenuated arthritis in the transgenic mice when assessed macroscopically and histologically, selectively in the delayed phase, after peak disease[23].

The present study made use of the serum-induced model of inflammatory arthritis to: i) monitor potential modulation of endogenous AnxA1, Fpr1 and Fpr2; ii) determine the effect of Dex in wild type and AnxA1$^{-/-}$ mice and; iii) establish the pharmacological potential of exogenously administered AnxA1.

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MATERIALS AND METHODS

Animals

BALB/c male 8-week old wild type AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice[24] were bred at Charles River (Kent, UK). All experiments were approved and performed under the guidelines of the Ethical Committee for the Use of Animals, Barts and the London School of Medicine and Home Office regulations (Scientific Procedures Act, 1986).

Model of Inflammatory Arthritis

Arthritis induction—Mice received either 50+50 μl (day 0 and 2) or 200 μl (day 0) of arthritogenic K/BxN serum.[22] Disease was monitored by assessing the clinical score where a maximum of 12 points could be given per animal: 0, no evidence of inflammation; 1 point, subtle inflammation on metatarsal phalanges joints, individual phalanx; 2 points, swelling on dorsal or ventral surface of paw; 3 points, major swelling on all aspects of paw. [25] Paw volume was assessed by water plethysmometry (Ugo Basile, Milan, Italy).

Pharmacological intervention—Mice were injected intraperitoneally with Dex (disodium salt; Sigma, Dorset, UK) prepared in sterile PBS (Sigma) and given at a dose-range of 3-30 μg once daily after disease onset (from day 2). AnxA1 and SuperAnxA1 were generated as recently described[26] and administered intraperitoneally, from day 2, at 1 μg per mouse.[26]

Histology and Immunohistochemistry

Histology—Joints were decalcified and paraffin embedded. Sections (4 micrometers) were stained with haematoxylin & eosin (H&E) or SafraninO with fast green counterstain. Standard light microscopy was used to determine the degree of synovitis, pannus formation, cartilage and bone erosion which were graded from 0 (no disease) to 5 (severe) by two blinded examiners, as reported.[22]

Immunohistochemistry—An antigen retrieval step using citrate buffer (pH6.0) was performed and then sections were incubated with rabbit polyclonal anti-AnxA1 (1:1000) (Zymed Laboratories, Cambridge, UK) or anti-FPR2 (1:4000).[27] After washing and the secondary biotinylated antibody (Dako, Cambridge, UK), staining was detected using a peroxidase conjugated streptavidin complex (Dako) and colour developed using DAB substrate (Sigma).

Molecular Analyses

Ankle joints were snap frozen in liquid nitrogen and homogenised in Qiagen RLT plus buffer (Qiagen, Crawley, UK) using Precellys24 ceramic bead homogenisation technology (Bertin Technologies, Montigny-le-Bretonneux, France). RNA was extracted using RNeasy® Plus mini kit (Qiagen) and genomic DNA contamination eliminated with Turbo DNA-free kit (Applied Biosystems, Foster City, USA). Complementary DNA was synthesised using SuperScriptIII reverse transcriptase and OligoDt primers (Invitrogen). Quantitative real-time PCR was performed using QuantiTect primers (Qiagen) and ABI Prism 7900 sequence detector system (Applied Biosystems). RQ values were calculated using the $2^{-(\Delta\Delta Ct)}$ method and normalised to an individual naïve AnxA1$^{+/+}$ or AnxA1$^{-/-}$ mouse (calibrator sample).

All Ct values were normalised to endogenous *Gapdh* gene product. Since factors such as hypoxia can influence *Gapdh* gene levels [28] a second house-keeping gene; *Rpl32* was used to normalise and confirm data (see Table S2).

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Statistics

Data are expressed as means ± SE. Student's *t* test was used to compare two groups. Comparison of clinical scores and paw volumes were made using 2-way ANOVA. A value of $P<0.05$ was considered significant.

RESULTS

Characterisation of joint inflammation

We began this study to optimise the model by comparing two different protocols: 200 μl of serum at day 0 (as in[22]) or 50+50 μl at day 0 and day 2. A remarkably similar arthritic response could be measured with respect to disease profile and intensity (Figure 1A). High levels of disease were achieved as early as day 2 post-injection with maximal response by day 6, after which disease resolved to almost normal by day 20. Incidence of disease was similar between the two volumes of K/BxN serum (Table S1). The arthritic response was mirrored by changes in paw volume with similar profiles with no difference between the two protocols (Figure 1B).

Microscopic analyses of non-arthritic ankle joints showed an acellular articular cavity with intact unicellular synovial lining and uniform cartilage structure (Figure 1C, top panel). In contrast, arthritic joints from both 50+50 μl and 200 μl K/BxN arthritis groups displayed marked cell infiltration within a thickened synovial lining. Pannus invasion leading to cartilage erosion was observed and proteoglycan loss was confirmed with SafraninO (Figure 1C, middle and bottom panels). Quantitative histological scoring showed no significant difference between the two groups of arthritic mice (Figure 1D).

The ankle is the predominant joint to be affected in this model of inflammatory arthritis, yet we monitored structural changes in the knee, as perhaps more relevant to human disease; we observed that knee joints displayed mild synovitis accompanied with thickening of synovial lining, pannus formation and marked cartilage erosion (see Figure S1). Based on these results, in agreement with a recent published study,[29] the 50+50 μl serum protocol was selected for subsequent experimentation.

Expression of AnxA1 and Fpr2 in the joint

At the mRNA level, a low degree of expression for both *Fpr1* and *Fpr2* could be measured, with high *Anxa1* message content (see Table S2). These results were partially confirmed by protein analyses, since AnxA1 was mildly expressed and localised predominantly in the synovial lining, whilst Fpr2 was localised to the periosteal lining with very little detection in synovium (Figure 2A). In arthritic joints, AnxA1 and Fpr2 expression was highly detectable within the synovial lining (Fpr2) and tissue (AnxA1 and Fpr2) (Figure 2B). Antibody controls and AnxA1$^{-/-}$ joints did not show any staining (Figure 2C and 2D).

Dexamethasone is inactive in AnxA1$^{-/-}$ mice

Despite wide application of this model of inflammatory arthritis, the bioactions of glucocorticoids have been scantly investigated. We selected the 10-μg dose of Dex from initial studies (Figure S2). Administration of Dex to AnxA1$^{+/+}$ mice exerted profound inhibitory effects with ~60% inhibition of the severity of arthritis at day 6 and ~80% at day 10 compared to vehicle-treated mice (Figure 3A). AnxA1$^{-/-}$ mice displayed a similar profile of arthritis to AnxA1$^{+/+}$ animals. However, the anti-arthritic effect of Dex was absent in animals nullified for AnxA1 (Figure 3A). Analysis of presence of severe disease (score ≥8) indicated a trend for higher incidence in AnxA1$^{-/-}$ mice and a marked reliance on endogenous AnxA1 for the protective effects of Dex (Table S3).

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Histological analyses were in line with arthritic scores. AnxA1$^{+/+}$ mice displayed marked synovitis accompanied by pannus formation and cartilage destruction (Figure 3B, left panels). Treatment with Dex prevented signs of disease (Figure 3B, middle panels), whilst AnxA1$^{-/-}$ mice treated with vehicle control (not shown) and Dex (Figure 3B, right panels) showed similar disease pathology to AnxA1$^{+/+}$ mice. These structural changes were reflected in the histological scores with ~75% inhibition afforded by Dex only in AnxA1$^{+/+}$ mice (Figure 3C).

Analyses of inflammatory gene messages, normalised to *Gapdh* and *Rpl32* (see Figure S3) and selected from our previous study [22], revealed that in wild type mice; Dex inhibited *Cxcl10*, *Nos2, Il-1β*, and *Ccl2*. *Tnfα* was inhibited by ~25% whilst expression of the chemokine receptor *Cxcr3* was not modulated (Figure 3D). These inhibitory effects of Dex were absent or significantly reduced in AnxA1$^{-/-}$ mice.

Next, we monitored expression of AnxA1 and its receptors, normalised to *Gapdh* and *Rpl32* (see Figure S4). In arthritic AnxA1$^{+/+}$ mice, both *Fpr1* and *Fpr2* mRNA expression was enhanced over naïve joints (Figure 4), a finding which is in line with Fpr2 protein expression (see Figure 2). Dex decreased both *Fpr1* and *Fpr2* message with little comparative effect on *Anxa1* (Figure 4). In mice devoid of AnxA1, a >10-fold increase in *Fpr1* expression was observed comparing arthritic to naïve joints, with *Fpr2* remaining at a similar expression. In absence of AnxA1, Dex could not regulate either *Fpr1* or *Fpr2* gene expression (Figure 4).

SuperAnxA1 accelerates resolution of the arthritic joint

In the final set of experiments we assessed the pharmacological potential of these findings. Proteinase 3 (PR3) may limit AnxA1 functions in neutrophilic inflammation, cleaving it at the N-terminal region. Thus we monitored the profile of *Pr3* gene, in the arthritic joint and found it significantly rose from baseline (day 0) to peak of disease (day 6) (Figure 5 and Figure 5S). This observation, coupled to abundant expression of Fpr1 and Fpr2 in the joints, justified the testing of SuperAnxA1. Dosed at 1 μg per mouse, AnxA1 was not particularly effective in reducing disease (Figure 5B). On the other hand, cleavage-resistant SuperAnxA1 did not affect disease progress (e.g. from day 2 to day 6) but significantly accelerated resolution (Figure 5B), with 50% less disease incidence compared to controls (Table 1).

The macroscopic joint protection afforded by SuperAnxA1 was confirmed microscopically, with preservation of cartilage structure with little loss of proteoglycans (Figure 5C, middle panels). Synovitis was apparent within these joints, but substantially lower than controls (Figure 5C, top panels). The joints of AnxA1 treated mice were disrupted to a similar extent as controls (Figure 5C, bottom panels). Quantitative analysis of damage within the ankle joints confirmed the observational studies (Figure 5D). Finally, ankle joints from vehicle- and SuperAnxA1-treated mice demonstrated reduced expression of the *Cxcl10* and *Cxcr3* pair, as well as *Nos2, Il-1β*, *Ccl2, Il-6* but not *Il-10* and *Tnfα* (Figure S6).

Discussion

AnxA1 is the prototype of a new class of mediators able to potently modulate both the innate and adaptive immune response; whilst effecting inhibitory properties on inflammatory cells, setting in motion proresolving responses, this protein acts as a positive relay in T cells activation.[4] As an example, exogenous and endogenous AnxA1 potentiate T cell receptor-downstream signalling [30] and assure proper dendritic cell-mediated T cell clonal expansion.[31] In agreement with these dual functions, AnxA1$^{-/-}$ mice experience augmented acute inflammatory responses,[14, 15] yet display reduced degree of tissue

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damage in an experimental model of multiple sclerosis.[32] Less clear is the role of the protein in models of arthritis.

In the collagen-induced arthritis model, AnxA1 injection during the immune phase (within week 1 post-collagen immunization) led to higher disease as emerged three weeks later.[30] In a model of antigen-induced arthritis, assessed at a single time-point (day 7), endogenous AnxA1 modulated some of the anti-inflammatory effects of Dex, including inhibition of pro-inflammatory genes including IL-1β and Fα.[20] No studies have yet investigated the role of endogenous or exogenous AnxA1 in the serum-induced inflammatory arthritis model.

K/BxN serum-induced arthritis,[33, 34] is a versatile model that can be applied to transgenic mice with little dependence on strain.[35] This model addresses the contribution of neutrophils and macrophages to inflammatory arthritis, as evident from depletion studies.[36, 37] Conversely, this experimental arthritis does not rely on T and B cells as demonstrated with the RAG1−/− mice[28] which, in contrast, display delayed disease onset and severity in collagen-induced arthritis.[38] Here we describe that low dosage arthritogenic serum provokes a high degree of arthritis and disease incidence, data in agreement with a recent study by Lee's group[29]. We noted a remarkable joint disruption and report a novel parallelism between the ankle and knee joint. Marked synovitis associated with disruption of cartilage occurs along a well-defined time-profile with peak disease at day 6 followed by a resolution phase.[22, 35] Use of transgenic mice has allowed some definition of the disease pathogenesis, revealing a fundamental role for myeloid cells[36, 37] and IL-1.[39, 40] More recently this model of arthritis has been applied to proresolving pathways, noting that 12/15-lipoxygenase or melanocortin type-3 receptor deficient mice experience a higher degree of disease.[21, 22]

AnxA1 is highly abundant in myeloid and stromal cells and its expression can be modulated by glucocorticoids and other anti-inflammatory drugs.[41] In addition, cell activation such as adhesion or extravasation also engages the AnxA1 system as a way to activate an inhibitory circuit to modulate the inflammatory response.[27] After several pharmacological investigations with AnxA1 or mimetics, AnxA1 null mice were produced and tested in models of acute inflammation[15, 24] revealing important modulatory functions.[4] As discussed above, AnxA1 role in chronic inflammatory settings in less defined.

This study began by detecting protein and mRNA of elements of the AnxA1 pathway during on-going inflammatory arthritis, reporting a high degree of expression for *Anxa1*, *Fpr1* and *Fpr2* both is resting and arthritic joints. At the protein level, Anxa1 localisation in the mouse synovium seems to parallel that observed in the human RA synovium[42]. However, at least in the conditions here applied, lack of AnxA1 does not seem to impact on the profile of disease. Yet it remains to be seen whether such a lack of function would also be seen in knockouts for the AnxA1 receptors.[5, 43] As an example, in preliminary observations, mice nullified for Fpr2 displayed higher arthritic score upon injection of the arthritogenic serum.[44]

Glucocorticoids have been scarcely tested in this model. Buttgereit and colleagues have tested the effect of osteoblast-restricted absence of glucocorticoid signalling on disease outcome, noting a worsening with marked joint disruption[23]. In these mice, bone cells could not respond to glucocorticoids from birth, thus it is possible that developmental aspects might be relevant and subtle long-term alterations occur. For instance it is know that glucocorticoids exert a permissive effect on importance cytokine function in the liver, by assuring proper expression of the IL-6 and IL-1 receptors[45, 46]. IL-6 is also a master-cytokine for osteoblasts (and fibroblasts) so a malfunctioning or inadequate glucocorticoid

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signalling, prolonged from birth, may generate responses that are not necessarily replicated by acute glucocorticoid treatment.

In keeping with this, our pharmacological analyses with doses of Dex as low as 10 μg exerted potent protective effects on the joint, evident both as inhibition of pannus formation and chondro-protection. It was striking to observe that the effects of the glucocorticoid were lost in AnxA1$^{-/-}$ animals. Thus, whilst AnxA1$^{-/-}$ mice did not experience a difference in the extent and profile of arthritis, at variance from the antigen-induced arthritis model,[20] endogenous AnxA1 still proved important in the inhibitory effects of Dex on a discrete set of proinflammatory genes, of which *Il-1β*, *Cxcl10* and *Ccl2* were of particular interest. Selective modulation of gene expression downstream AnxA1 is in keeping with the observations made in the antigen-induced arthritis model.[20] The fundamental role that IL-1β exerts in this model of inflammatory arthritis has been addressed through a variety of genetic approaches targeting directly the cytokine, its receptor[39, 47] or endogenous antagonist.[40]

Modulation of gene expression downstream of endogenous AnxA1 can also be discussed in relation to the modulation of genes of the AnxA1 pathway. In fact, Dex did not appear to induce *Anxa1* or *Fpr* gene expression at the level of the joint, yet was highly reliant on endogenous AnxA1 both with respect to cell recruitment and inflammatory gene expression. We reason that these data indicate multiple functions of endogenous AnxA1, which become of relevance only after administration of the glucocorticoid.

The lack of correlation between AnxA1 gene product and protein is intriguing though It should be said that glucocorticoid-regulation of the AnxA1 pathway is multifaceted; thus, non-genomic modulation[48] with release of the protein, for instance, could account for apparent lower tissue levels of the AnxA1 protein. AnxA1 analysis, at a single time point, could also be confounded by genomic regulation exerted by glucocorticoids[15, 49] and other factors present in an inflamed joint such as IL-6[50] and TNF[51]. Clearly, the current lack of characterisation of the AnxA1 promoter contributes to this poor understanding of the mechanisms operative in these settings. Moreover, the fact that glucocorticoids like dexamethasone induce AnxA1 gene in innate immune cells [14, 15] whilst downregulating it in adaptive immune cells[52] add another layer of complexity to this issue which inevitably requires further experimentation.

The versatility of this model of inflammatory arthritis justifies its application to a variety of targets; in line with the major infiltration of neutrophils and mast cells,[53] we could observe dynamic expression of *Pr3* gene product in the arthritic joint. Proteinase 3 is emerging as an important effector of neutrophilic inflammation[54] and can cleave, among a variety of substrates, also AnxA1.[55] Therefore, we concluded this study by testing the potential of AnxA1 mimetics, comparing the effect of native protein with that of SuperAnxA1, a mutant resistant to serine protease-induced cleavage.[26] Administered at the dose of 1 μg (corresponding to 27 pmol), SuperAnxA1, but not AnxA1, attenuated joint disease. It was interesting to note that SuperAnxA1 did not impact on the induction of arthritis yet it accelerated the resolution process so that by day 10 a preserved joint structure could be observed. We wish to propose that higher resistance to proteolysis might underlie its pharmacological efficacy, though we cannot exclude the confounding element of potential differences in pharmacokinetics. In any case, these results provide strong proof-of-concept that an AnxA1 derivative, resistant to proteolysis, could be beneficial in modulating joint inflammation. SuperAnxA1 could be the backbone for future strategies aiming at capitalising AnxA1 biology for novel anti-arthritic approaches.

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Glucocorticoids are of wide clinical use because of their ability to impact several effectors of arthritis, with a recent appreciation that, besides inhibition of proinflammatory gene expression, gene induction is also of therapeutic relevance.[56, 57] In the context of chronic inflammatory diseases, induction of dual-specificity phosphatase 1[58] and glucocorticoid-induced leucine zipper[59] genes could be of mechanistic value. In addition, we show here that endogenous AnxA1 is also relevant to the anti-arthritic effect of low-dose Dex, yet we cannot demonstrate induction of this gene at the level of the arthritic joint leading to our proposal that the protein is either modulated in a non-genomic fashion by this glucocorticoid[41, 60] or the regulation occurs outside the site of the arthritic joint, an obvious site being the vasculature and the control of the egress of blood-borne neutrophils[4]. Analysis of AnxA1 expression in distant tissues (e.g. blood) during on-going arthritis and glucocorticoid treatment can shed light on this point, which has also been touched upon above in relation to lack of correlation between joint AnxA1 mRNA and protein expression.

Collectively, this study reveals potent anti-arthritic properties for Dexamethasone in a model of inflammatory arthritis, using very low doses perhaps reminiscent of clinical application on low-dose glucocorticoids in rheumatoid arthritis[61] and, in terms of drug discovery programmes, justifies the testing of AnxA1 mimetics to control the active phase of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding This work was supported by a Wellcome Trust (UK) project grant 083551. SMO is funded Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (Grant 2011/00128-1) and Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Grant 302768/2010-6)

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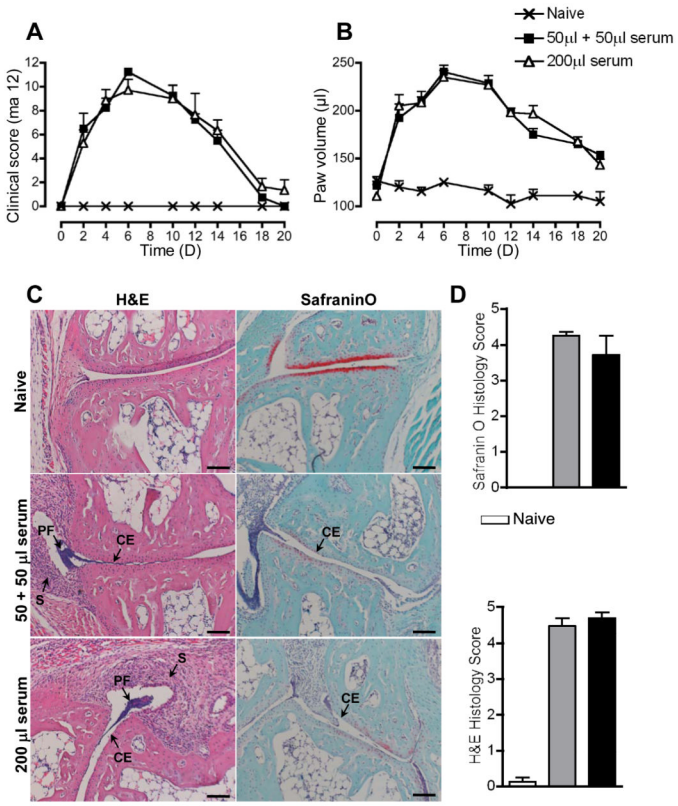

Figure 1. Characterisation of the arthritic response to low volume K/BxN serum

Mice (n=8) were given an i.p. injection of 50 µl at day 0 and day 2 (50+50 µl) or a single injection of 200 µl at day 0 of K/BxN serum. (**A**) Arthritic scores and (**B**) paw edema were monitored over the time course. No significant difference (two-way ANOVA) was observed between the two K/BxN serum groups. (**C**) Ankles of naïve, 50+50 µl and 200 µl K/BxN serum group. Decalcified paraffin embedded joints were staining by H&E and SafraninO. Representative images are shown; scale bars, 50 µm. (**D**) Histomorphometric analyses of joint sections; S, synovitis; CE, cartilage erosion; PF, pannus formation. All results are expressed as mean ± SE.

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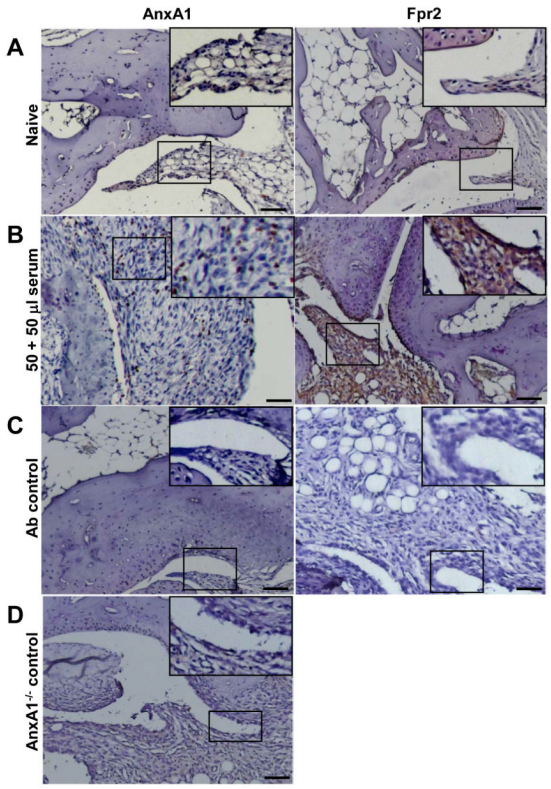

Figure 2. Protein expression of AnxA1 and Fpr2 in ankle joints of naïve mice

Ankles of naïve and K/BxN serum (50+50 µl, day 0 and day 2) were processed (see Methods) and stained for AnxA1 and Fpr2, with a haematoxylin counterstain. Images representative of three distinct analyses in 4 mice per status are shown. (**A**) Naïve mouse ankle joints. (**B**) Serum-treated mouse ankle joint. (**C**) Same joints but tested without the primary antibody. (**D**) Joint of an AnxA1$^{-/-}$ mouse, acting as negative control for AnxA1 staining. Scale bars, 20 µm.

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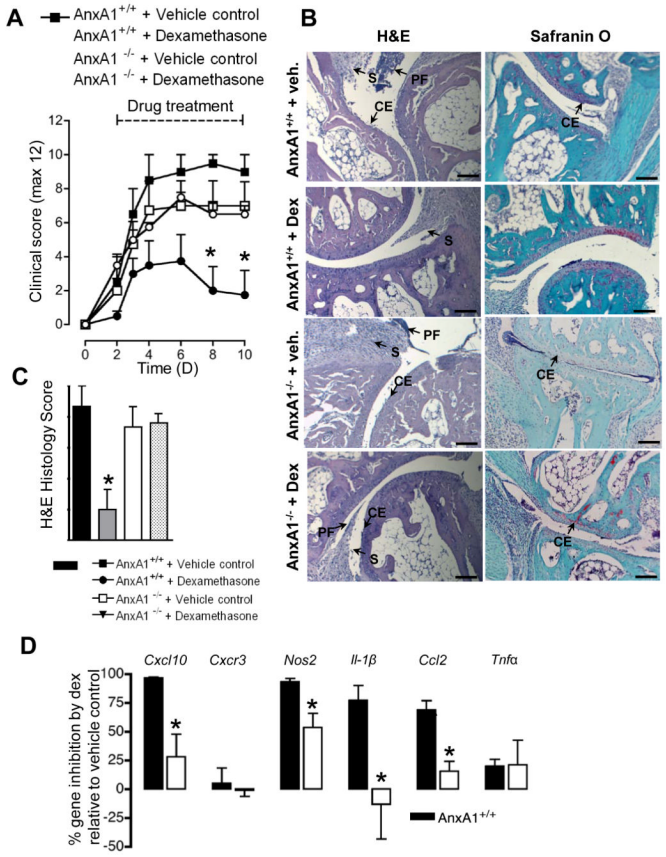

Figure 3. Dexamethasone attenuates K/BxN arthritis in AnxA1$^{+/+}$ but not AnxA1$^{-/-}$ mice

AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice were given an intraperitoneal injection of K/BxN serum (50 μl at day 0 and day 2) and then received vehicle or Dex (10 μg i.p. daily). (**A**) Arthritic scores as monitored over a 10-day time course. *p<0.05 *vs.* appropriate vehicle control (two-way ANOVA). (**B**) Right ankles of mice from each experimental group were taken at day 10 and joints processed for staining by H&E and SafraninO. Representative images are shown. Scale bars, 50 μm. (**C**) Histomorphometric analyses of joint sections; S, synovitis; CE, cartilage erosion; PF, pannus formation. *p<0.05 *vs.* appropriate vehicle control (Student's *t* test). (**D**) Quantitative real-time PCR was performed on cDNA from left ankle joints; proinflammatory genes were analysed with all Ct values normalised to endogenous *Gapdh* (4 mice per group). RQ values were calculated using $2^{-(\Delta\Delta Ct)}$ and data shown here as mean % gene inhibition by Dex relative to vehicle control groups ± SE. Naïve joints were set as the calibrator samples. *p<0.05 *vs.* respective vehicle control (Student's *t* test).

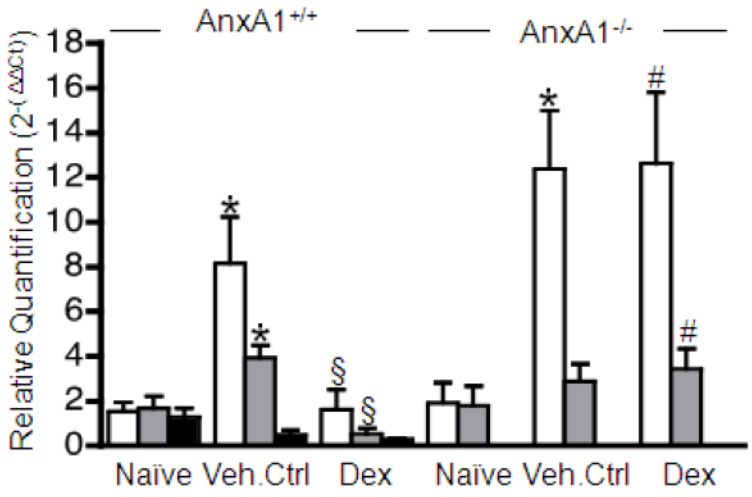

Figure 4. Profile of *Anxa1*, *Fpr1* and *Fpr2* gene product expression in ankle joints of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice treated with Dex

AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice were given an intraperitoneal injection of K/BxN serum (50 μl at day 0 and day 2) and then received vehicle or Dex (10 μg i.p. daily). Quantitative real-time PCR was performed on cDNA from left ankle joints of mice from each experimental group (Day 10). The *Anxa1*, *Fpr1* and *Fpr2* genes were analysed with all Ct values normalised to endogenous *Gapdh* (4 mice per group). Naïve joints were set as the calibrator samples. Mean RQ values ± SE shown here were calculated using $2^{-(\Delta\Delta Ct)}$ method. *p<0.05 *vs.* naïve; §p<0.05 *vs.* appropriate vehicle control; $^{\#}$p<0.05 *vs.* AnxA1$^{+/+}$ group (Student's *t* test).

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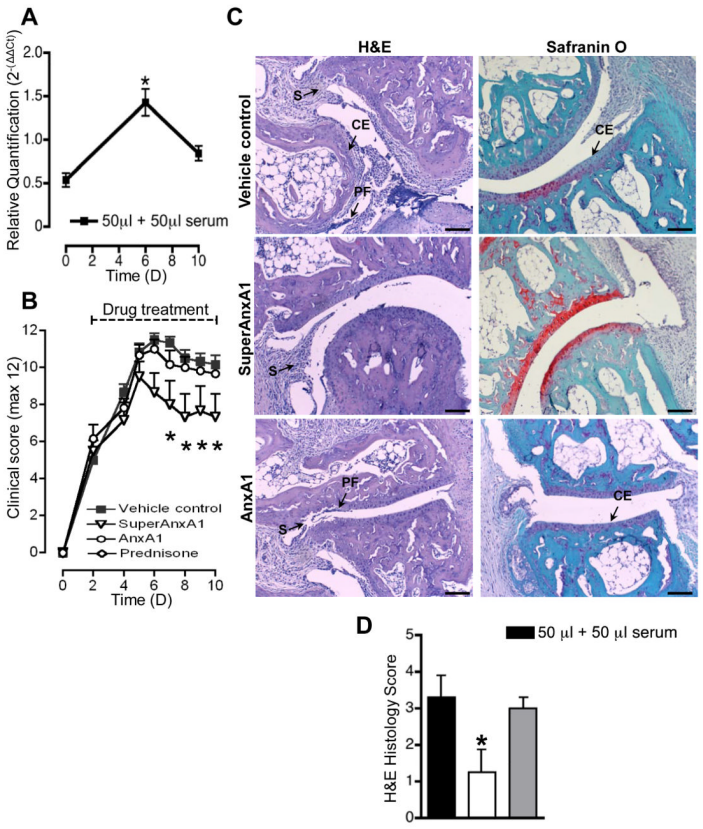

Figure 5. SuperAnxA1 accelerates resolution of K/BxN arthritis

AnxA1$^{+/+}$ mice (n=6) were given an i.p. injection of K/BxN serum (50 μl at day 0 and day 2) and then received vehicle, human recombinant AnxA1 or SuperAnxA1 (1 μg i.p. daily from day 2). (**A**) *Pr3* gene product expression was analysed with Ct values normalised to endogenous *Gapdh*. RQ values were calculated using $2^{-(\Delta\Delta Ct)}$ and data shown here as mean ± SE. Naïve mice (D0) were set as the calibrator samples. *p<0.05 vs. naïve (Student's *t* test). (**B**) Arthritic score. *p<0.05 *vs.* vehicle control (two-way ANOVA). (**C**) Right ankles of mice from each experimental group were taken at day 10 and joints processed for staining by H&E and SafraninO. Representative images are shown. Scale bars, 50 μm. (**D**) Histomorphometric analyses of joint sections; S, synovitis; CE, cartilage erosion; PF, pannus formation. *p<0.05 vs. vehicle control (Student's *t* test).

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Table 1

SuperAnxA1 reduces the severity of inflammatory arthritis.

Time (days)	Vehicle control	SuperAnxA1	AnxA1
0	0% (0/6)	0% (0/6)	0% (0/6)
6	100% (6/6)	83.3% (5/6)	100% (6/6)
10	100% (6/6)	50% (3/6)	100% (6/6)

Data report the number of mice with a severe arthritic score, taking a threshold of ≥ 8. SuperAnxA1 or AnxA1 were given from day 2 at daily doses of 1 μg i.p.; the K/BxN arthritogenic serum was given on day 0 and day 2 at volumes of 50 μl each time.