

Original article

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Assessment of murine collagen-induced arthritis by longitudinal non-invasive duplexed molecular optical imaging

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Abstract

Objective. In the present study we evaluated the use of four commercially available fluorescent probes to monitor disease activity in murine CIA and its suppression during glucocorticoid therapy.

Methods. Arthritis was induced in male DBA/1 mice by immunization with type II collagen in Complete Freund's Adjuvant, followed by a boost of collagen in PBS. Four fluorescent probes from PerkinElmer in combination [ProSense 750 fluorescent activatable sensor technology (FAST) with Neutrophil Elastase 680 FAST and MMPsense 750 FAST with CatK 680 FAST] were used to monitor disease development from day 5 through to day 40 post-immunization. Fluorescence generated *in vivo* by the probes was correlated with clinical and histological score and paw measurements.

Results. The fluorescence intensity emitted by each probe was shown to correlate with the conventional measurements of disease. The highest degree of correlation was observed with ProSense 750 FAST in combination with Neutrophil Elastase 680 FAST; these probes were then used to successfully assess CIA suppression during dexamethasone treatment.

Conclusion. We have demonstrated that longitudinal non-invasive duplexed optical fluorescence imaging provides a simple assessment of arthritic disease activity within the joints of mice following the induction of CIA and may represent a powerful tool to monitor the efficacy of drug treatments in preclinical studies.

Key words: collagen-induced arthritis, optical imaging, ProSense 750 FAST, neutrophil elastase 680 FAST, MMPsense 750 FAST, CatK 680 FAST, dexamethasone.

Rheumatology key message

- Longitudinal non-invasive optical fluorescence imaging is a powerful tool for the assessment of murine CIA.

Introduction

Non-invasive optical bioimaging techniques are of increasing interest as a means of improving both

preclinical research and clinical diagnostics or monitoring of arthritic disease [1, 2]. Currently there are a variety of imaging agents available that may be used to monitor disease activity. The simplest are perhaps non-specific agents such as the Indocyanine dyes, which injected intravenously, can be used to monitor inflammation as a result of accumulation at sites of increased blood flow and vascular leakage. Indocyanine dyes have been used preclinically in rodent arthritis models [3, 4] and clinically in RA patients where accumulation of Indocyanine Green fluorescence has been shown to correlate with both MRI and US assessments of disease severity [5–7]. Imaging of this nature offers a technically simple and relatively inexpensive means of monitoring disease activity. More

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information, however, can be obtained about the underlying disease mechanism by using fluorescent probes that are targeted to specific disease-associated molecules or processes: for example, conjugation of fluorochromes to antibodies against F4/80 (a marker for macrophages) [8] or E-selectin (a marker of endothelial cell activation) [9], or the labelling of small molecules such as folate, the accumulation of which has been associated with the increased metabolic activity observed at sites of inflammation [10]. Reactive probes may also be used to monitor the production of reactive oxygen species [11] or the activity of inflammation-associated enzymes such as cathepsins [12–15] and MMPs [16–20]. Probes such as luminol, which react with enzymes that are produced by specific cell types, may also be used to monitor the infiltration and activity of those cells into sites of inflammation [21]. More recently, cell death was assessed in CIA by using a near-infrared PSVue 794 dye [22].

In addition to monitoring inflammatory or autoimmune conditions, activatable fluorescent probes have been used successfully to monitor disease and therapeutic efficacy in a variety of cancer models. MMPsense 680 fluorescence has been shown to correlate with the presence of tumours *in vivo* [23], while Neutrophil Elastase 680 has been used to monitor neutrophil activity within tumours following photodynamic therapy [24]. Decrease in bone metastasis-associated CatK 680 fluorescence has been shown to correlate with successful treatment in a murine model of breast cancer [25].

Despite one of the major advantages offered by non-invasive imaging being the ability to repeatedly evaluate the molecular processes occurring within arthritic joints over the entire course of the experimental model, the majority of studies to date have only looked at one or two time points, and in addition only evaluate a single reagent in each animal. In the present study, we show the use of four commercially available fluorescent probes from PerkinElmer in combination [ProSense 750 fluorescent activatable sensor technology (FAST) with Neutrophil Elastase 680 FAST; and MMPsense 750 FAST with CatK 680 FAST] to monitor disease development in the CIA model in mice from day 5 through to day 40 post immunization. These probes are activatable fluorescent imaging agents comprising a novel architecture (termed FAST) that confers an improved pharmacokinetic profile with earlier imaging time points. These probes are optically silent in their naïve state and become highly fluorescent following enzyme-mediated activation. ProSense 750 FAST is activated by proteases such as cathepsin B, L, S, K, V and D. Neutrophil Elastase 680 FAST produces a fluorescent signal after cleavage by elastase produced by neutrophils. MMPsense 750 FAST is a MMP-activatable agent that produces fluorescent signal after cleavage by a broad range of MMP's including MMP 2, 3, 7, 9, 12 and 13. CatK 680 FAST produces fluorescent signal after cleavage by cathepsin K.

The fluorescence intensity (FI) emitted by each probe was shown to correlate with the conventional measurements of disease. The highest degree of correlation was

observed with ProSense 750 FAST in combination with Neutrophil Elastase 680 FAST. These probes were then used to assess the anti-inflammatory effect of dexamethasone.

Methods

Mice

Male DBA/1 mice were obtained from Harlan (Bicester, UK). All mice were used at 6–7 weeks old and maintained at the Biological Services Unit, University of Glasgow, in accordance with Home Office UK guidelines.

Induction of CIA

On day 0, CIA was induced under light anaesthesia in DBA/1 male mice by intradermal injection of 200 µg Bovine Type II Collagen (MD Biosciences, St Paul, MN, USA) emulsified in Complete Freund's Adjuvant (Chondrex, Redmond, WA, USA) into the shaved rump, followed by a boost on day 21 using 200 µg of Bovine Type II collagen in PBS administered intraperitoneally [26].

Clinical scoring regime

Signs of arthritis were assessed in all paws according to a 0–4 scale of ascending severity: 0 = no reaction; 1 = mild, but definite redness and swelling of the ankle/wrist or apparent redness and swelling limited to individual digits, regardless of the number of digits affected; 2 = moderate to severe redness and swelling of the ankle/wrist; 3 = redness and swelling of the entire paw including digits; and 4 = maximally inflamed limb with involvement of multiple joints [26]. Paw thickness as a measure of swelling was determined in millimetre three times weekly using dial callipers (Kroeplin, Munich, Germany) [27]; data are presented as the mean thickness of all four paws. All animals were scored and the paw measurements were taken on the day of imaging.

Probe administration protocol

Four fluorescent probes were used in this study: Neutrophil Elastase 680 FAST, ProSense 750 FAST, CatK 680 FAST and MMPsense 750 FAST (all from PerkinElmer, Llantrisant, UK). Probes were administered as cocktails: Neutrophil Elastase 680 FAST together with ProSense 750 FAST, and CatK 680 FAST with MMPsense 750 FAST. The absence of significant bleed-through between probes was previously determined (supplementary Fig. S1, available at *Rheumatology* Online). Probe cocktails were administered intravenously on days 5, 19, 27, 33 and 40 to naïve ($n = 3$) and CIA-induced ($n = 6$) animals. An additional group of animals were imaged without the administration of probe ($n = 3$). Probes were administered at a dose of 4 nmol/25 g mouse according to the manufacturer's instructions.

In a separate experiment, Neutrophil Elastase 680 FAST and ProSense 750 FAST were administered intravenously on days 28 and 35 to CIA-induced animals treated intraperitoneally from day 21 with either the vehicle (5% ethanol in PBS) ($n = 6$) or dexamethasone (Sigma-Aldrich,

Dorset, UK) at 1 mg/kg/day (n=6). An additional group of animals were imaged without the administration of probe (n=3). Following imaging on day 35, mice were culled and the rear paws collected and fixed in neutral buffered formalin.

In vivo optical imaging

Images were acquired on a Kodak *in vivo* imaging system FX Pro (Carestream, Hertfordshire, UK). Animals were anaesthetised using isoflurane and oxygen, and fur removed from the limbs using a depilatory cream (Boots Pharmacy Ltd, UK). Black electrical insulating tape was used to cover the abdomen to reduce non-specific signal from probes degraded during excretion, and the mouse was placed in the prone position in the imaging unit. Imaging was performed 8 h following intravenous administration of Neutrophil Elastase 680 FAST and ProSense 750 FAST and 6 h following the administration of CatK 680 FAST and MMPsense 750 FAST. Band pass excitation filters of 630 (10) nm were used for Neutrophil Elastase 680 FAST and CatK 680 FAST and of 730 (10) nm for ProSense 750 FAST and MMPsense 750 FAST. Emission filters were 700 (17.5) nm and 790 (17.5) nm, respectively. The entire acquisition time per animal was ~10 min. The exposure time was 90 s for Neutrophil Elastase 680 FAST and CatK 680 FAST, with 1 × 1 pixel binning and an aperture f-stop of 2.51. The exposure time for ProSense 750 and MMPsense 750 FAST was 129 s, with 1 × 1 pixel binning and an aperture f-stop of 2.51. X-Ray images were taken using a 0.4-mm aluminium filter with an exposure time of 60 s, 1 × 1 pixel binning and an aperture f-stop of 3.99. Fluorescent images are displayed as pseudocoloured, overlaid on the X-ray images. Scaling was determined manually and applied by the imaging software to the acquired grey-scale fluorescent images.

Image analysis

Image analysis was performed using the Carestream Molecular Imaging software (Carestream Health, Inc., Rochester NY, USA). A region of interest (ROI) tool was used to carefully draw around each paw, forming four ROIs per mouse. The background fluorescence signal was determined on a blank area of the image, and the mean background pixel FI was subtracted from the ROI pixel FI. The total FI (in arbitrary units) for each ROI was then determined by the software. Fluorescence data are presented as the Total FI for all paws per mouse or the Total rear paw FI (for comparison with histopathology data only).

Histological scoring

Rear paws were sampled on day 35, fixed in neutral buffered formalin (CellPath Ltd, Powys, UK), decalcified in EDTA, stained with haematoxylin and eosin or toluidine blue (Sigma-Aldrich) [28], and scored in a blinded fashion, as described below [26]. Data is presented as the mean of the sum of the scores obtained for the three parameters obtained for each paw.

For the inflammatory cell infiltrates, the scoring system was as follows: 0=no infiltrate detected; 1=modest leucocyte infiltration in synovial tissue, no fluid leucocytes; 2=moderate leucocyte infiltration in synovial tissue and in fluid phase, with loss of synovial architecture; 3=gross leucocyte infiltration in synovial membrane and fluid space, with significant loss of synovial and articular architecture. For synovial hyperplasia, the scoring system was as follows: 0=no abnormalities detected; 1=synovial lining layer 2–4 cells thick; 2=synovial lining layer >5 cells thick associated with moderate expansion of the sub-lining layer zone; 3=synovial lining layer >5 cells thick associated with significant expansion of the sub-lining layer zone and potentially with loss of synovial architecture. For erosion of cartilage and bone: 0=no abnormalities; 1=fibrillation of cartilage and/or mild erosive infiltration of periosteal and subchondral bone, nuclei intact within lacunae; 2=moderate fibrillation and loss of cartilage and/or moderate erosive infiltration of periosteal and subchondral bone; 3=significant loss of cartilage and/or erosive infiltration of periosteal and subchondral bone, nuclei show apoptosis within lacunae across a wide area of cartilage/bone.

Statistical analysis

Where appropriate, data are expressed as the group mean (standard error of the mean). Statistical significances were determined by Students t-test using the Winstat add-in for Excel. A $P < 0.05$ was taken to be significant. Correlations between the arthritis score, paw measurements, histopathology score and FI were performed using the Spearman Rank Correlation Analysis. A $P < 0.05$ was deemed to be significant.

Results

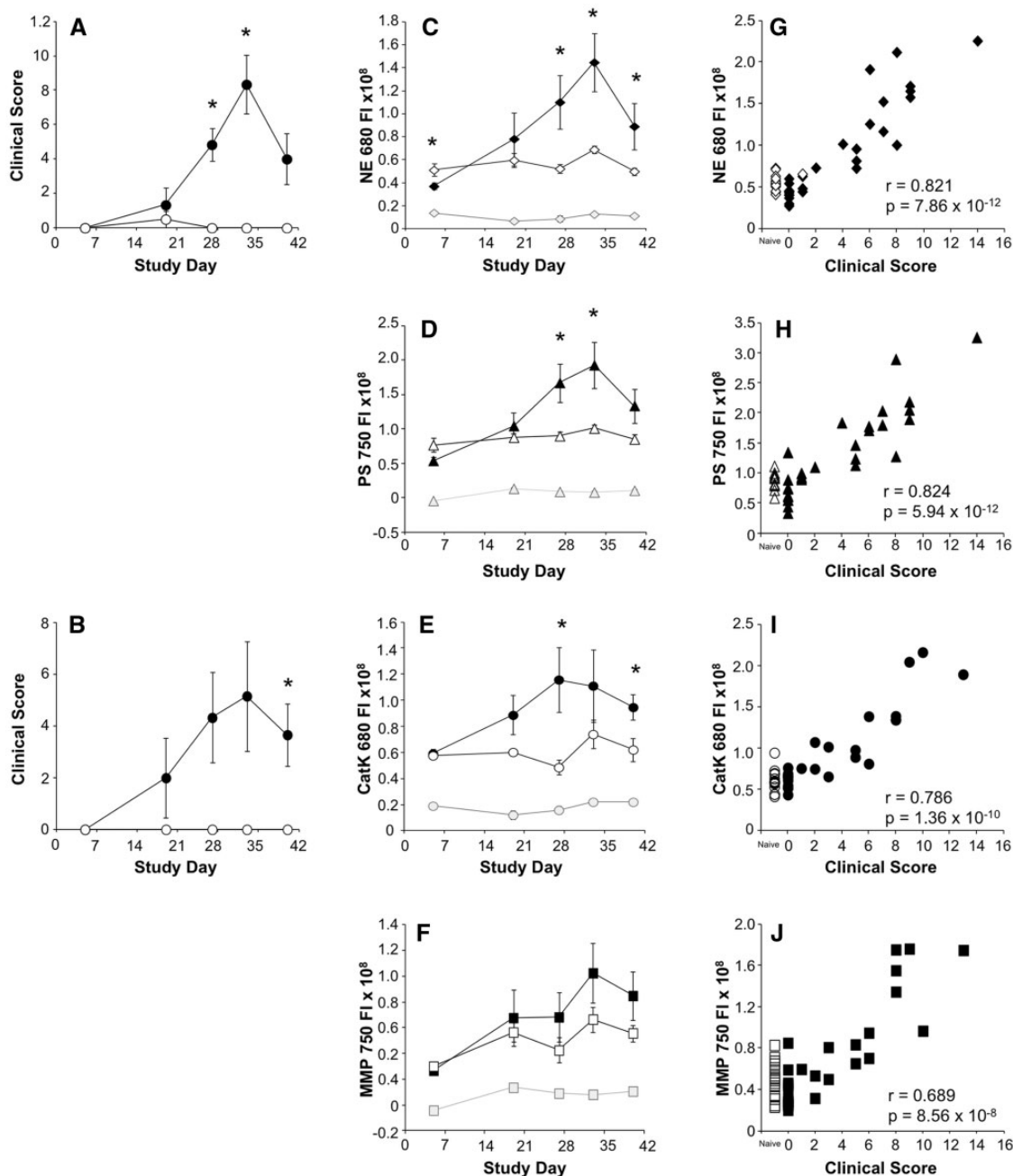
Fluorescence from commercially available activatable probes correlates with clinical disease score in CIA

As is typically observed in this model, very little disease was observed prior to the second collagen injection on day 21; following this, the clinical score increased, peaking on day 33 (Fig. 1A and B). The naïve groups (n=3) remained disease free throughout the study, except for a single animal in the Neutrophil Elastase 680 FAST/ProSense 750 FAST-treated group, which developed a transient score of 1, which was probably a minor injury and consistent with normal activity.

The increase in clinical score observed in the CIA mice was mirrored by the FI from the neutrophil elastase- and cathepsin- (both the multi-cathepsin-sensitive ProSense and the cathepsin K-specific CatK) sensitive probes (Fig. 1C–E). MMPsense gave the more variable fluorescence in both the naïve and the arthritic animals (Fig. 1F).

Neutrophil Elastase 680 FAST and ProSense 750 FAST were the more sensitive probes, giving the highest degree of correlation between total clinical score and total FI (Fig. 1G and H). CatK 680 FAST FI, while mirroring the disease induction, was a little less sensitive, showing a lower degree of correlation with the clinical score; the

Fig. 1 Fluorescence from the activatable probes correlate with disease score in CIA



(A) The clinical arthritis score for naïve mice ($n=3$) (black line open shape) and CIA mice ($n=6$) (black line closed shape) treated with the Neutrophil Elastase 680 FAST and ProSense 750 FAST cocktail. **(B)** The clinical arthritis score for naïve mice ($n=3$) and CIA mice ($n=6$) treated with the CatK 680 FAST and MMPsense 750 FAST cocktail. * $P < 0.05$. **(C–F)** Fluorescence intensity detected within the fore and rear paws of CIA mice (black line closed shape) or naïve mice (black line open shape) following the administration of a Neutrophil Elastase 680 FAST **(C;** diamond) and ProSense 750 FAST cocktail **(D;** triangle) or a CatK 680 FAST **(E;** circle) and MMPsense 750 FAST **(F;** square) cocktail. Images were also acquired at the relevant wavelengths in mice without probe treatment ($n=3$) (grey line open shapes). * $P < 0.05$. Graphs **(G–J)** The correlation of the total fluorescence intensities and the total clinical arthritis score throughout the study. Correlation coefficient (r) and P -values are shown on the graphs. FAST: fluorescent activatable sensor technology.

increase in fluorescence above that observed in the naïve animals was only evident in mice with a total score of >6 (Fig. 1I). This probably relates to the degree of disease required in the CIA model before bone erosion, resulting from increased osteoclast (and therefore cathepsin K) activity, develops [29, 30]. The lowest degree of correlation with clinical arthritis scores was observed with the fluorescence from the MMPsense 750 probe, with an increase in fluorescence above that observed in the naïve animals only observed in mice with a total score of >8 (Fig. 1J).

The probes Neutrophil Elastase 680 FAST and ProSense 750 FAST can be used to monitor the efficacy of an anti-inflammatory drug treatment in the CIA model

The highest degree of correlation between score and fluorescence was observed with the Neutrophil Elastase 680 FAST and ProSense 750 FAST probes. These two probes were therefore carried forward to test the use of non-invasive imaging for monitoring the efficacy of the anti-inflammatory drug, dexamethasone (1 mg/kg/day, starting on day 21) in the CIA model.

As previously observed, only mild disease in a few animals developed prior to the boost on day 21; subsequent to the boost, clinical scores increased in the vehicle-treated animals and continued to do so until the experiment terminated. In the dexamethasone-treated mice, the mild disease that was observed prior to day 21 resolved, and the mean clinical score had returned to 0 by day 27, remaining constant until termination (Fig. 2A). Paw thickness was also measured and this closely mirrored the clinical scores, with dexamethasone significantly reducing paw thickness from day 27 until the end of the experiment compared with the vehicle-treated group (Fig. 2B). Significant correlation was also observed between the clinical scores and paw thickness measurements (Fig. 2C).

The probe cocktail was administered on days 28 and 35 (prior to and at the disease peak), and the mice were imaged after 8 h. The fluorescence intensities generated by Neutrophil Elastase 680 FAST and by ProSense 750 FAST in the dexamethasone-treated CIA mice was significantly reduced compared with the vehicle-treated control animals at both 28 and 35 days (Fig. 2D and E). Arthritic paws are clearly visible in the pseudocoloured fluorescent image and X-ray overlays from the vehicle-treated mice and absent from the dexamethasone-treated animals for both Neutrophil Elastase 680 FAST and for ProSense 750 FAST (Fig. 2F). In addition to the paws, there are fluorescent hot spots apparent in the knee joints of some of the vehicle-treated animals (#1, 2, 4 and 6). This suggests the presence of inflammation within the knee joint, which is something that is not possible to score clinically. An additional bright spot was observed in mouse 4 over the abdomen; this signal was due to the urinary excretion of the probe localized in the bladder. The fluorescence intensities in the paws for both probes were found to correlate significantly with both the clinical score (Fig. 2G and H) and with paw thicknesses (Fig. 2I and J).

Dexamethasone reduced the clinical score by almost 10 points at 35 days, and the treated mice displayed almost no signs of arthritis. This is not always the case when new drugs are tested in the CIA model. We therefore analysed whether the fluorescent signal from the Neutrophil Elastase FAST 680 and ProSense FAST 750 probes have the potential to detect even small differences between treatment groups. To address this issue, data were pooled from the control CIA mice taken from two experiments at two different time points (4 and 5 weeks) following primary type II collagen immunization (difference in clinical score of 4.22). Analysis of the data demonstrated that, although less sensitive than the clinical score, these probes have the potential to detect small differences between groups (supplementary Fig. S2, available at *Rheumatology* Online).

Correlation with histopathological scores

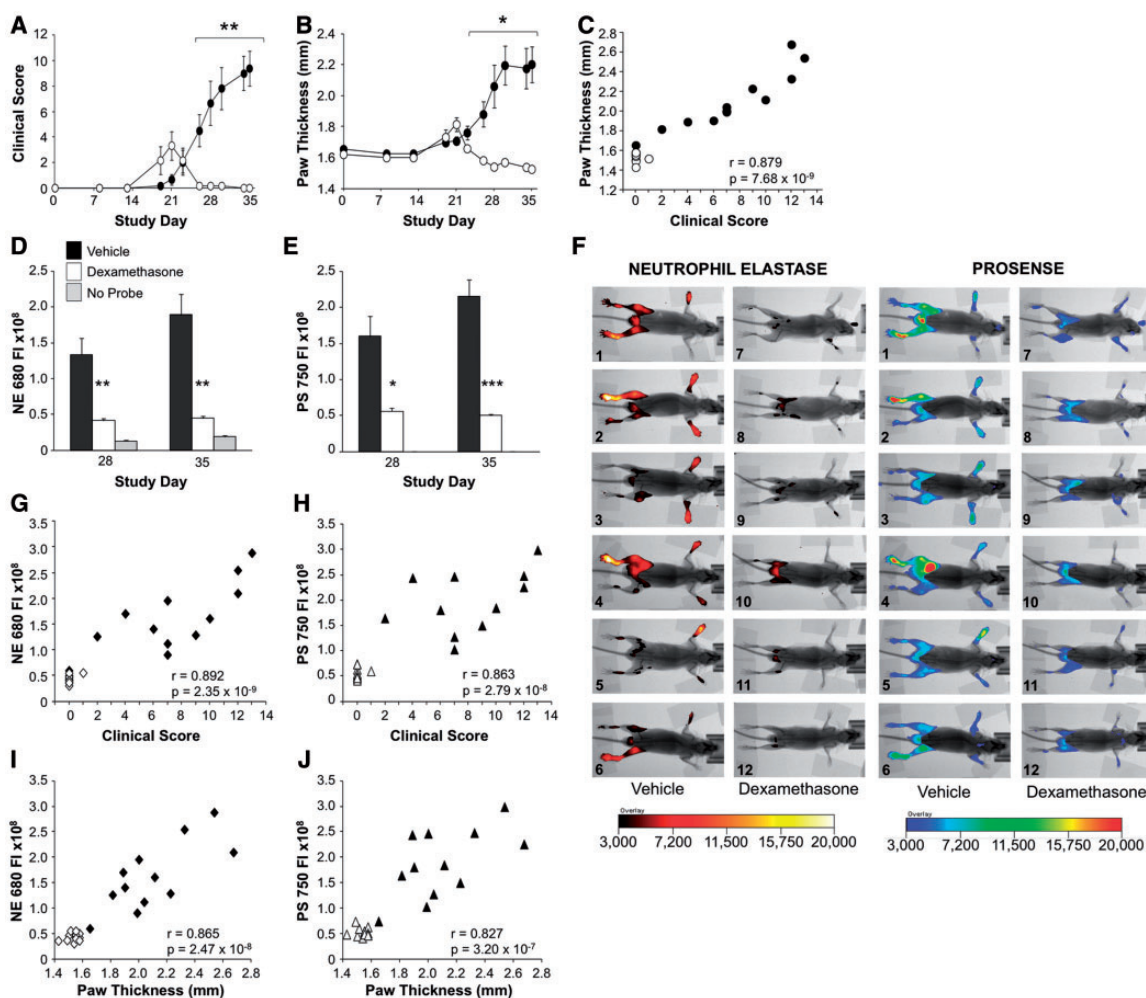
As a gold standard measure of disease activity in preclinical arthritis studies, the histological scoring data obtained from rear paws sampled at day 35 was also compared with the fluorescence imaging data. The histopathology score in dexamethasone-treated animals was found to be significantly lower than that observed in the control vehicle-treated animals (Fig. 3A). The rear paw histopathology scores were then compared with the rear paw thickness, rear paw clinical scores and with the rear paw FI from both probes. The clinical scores and paw measurements correlated with the histopathology scores (Fig. 3B and C, respectively); a similar degree of correlation was also observed between the FI obtained from Neutrophil Elastase 680 FAST and ProSense 750 FAST (Fig. 3D and E, respectively).

Discussion

Increased activity of proteases such as the cathepsins [31] and MMPs [32] and infiltration of inflammatory cells such as neutrophils [33] are associated with a variety of autoimmune and inflammatory conditions, including RA. Here we demonstrate the use of four commercially available probes to detect increased activity of cathepsins (ProSense 750 FAST or CatK 680 FAST), MMPs (MMPsense 750 FAST) and neutrophil elastase (Neutrophil Elastase 680 FAST) in the arthritic paws of mice following the induction of CIA, albeit with varying degrees of sensitivity.

The probes Neutrophil Elastase 680 FAST and ProSense 750 FAST (the latter activated by multiple cathepsins) showed the greatest ability to distinguish mildly diseased paws from normal paws. These results are consistent with data showing increases in gene expression and enzyme activity [34, 35] or the role of neutrophils in the development of arthritis. Our data also support previous findings demonstrating the ability of ProSense to detect cathepsin activity *in vivo* in a K/BxN serum-transfer model of arthritis [14, 15]. In our hands, MMPsense 750, a probe with multiple MMP sensitivities showed a relatively high degree of variability and was only able to detect differences between naïve and CIA animals

Fig. 2 Dexamethasone treatment of CIA mice reduces the fluorescent signal obtained from both the neutrophil elastase-sensitive and the cathepsin-sensitive probe

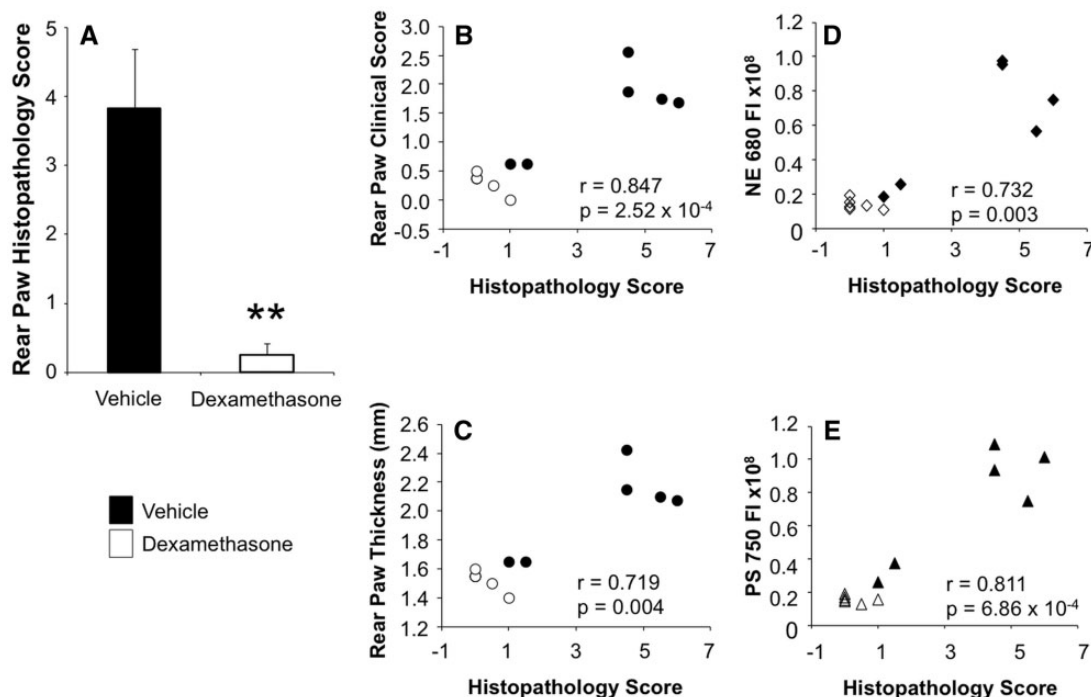


(A) The total clinical score; (B) shows the mean paw thickness determined throughout the study; and (C) shows the correlation between the clinical score and the paw thickness at days 28 and 35 in CIA mice treated with either the vehicle control (black) or dexamethasone (white). (D and E) Fluorescence intensity for Neutrophil Elastase 680 FAST and ProSense 750 FAST detected within the fore and rear paws of CIA mice treated with dexamethasone ($n = 6$) or vehicle ($n = 6$) on days 28 and 35 post-immunization (black columns, Vehicle; white columns, Dex; grey columns, no probe). (F) Fluorescence-X-ray overlay images obtained on day 35 from CIA mice treated with either dexamethasone or the vehicle control. The two panels show the same animals. (G–J) The correlation of the Neutrophil Elastase 680 FAST and ProSense 750 FAST FI with the clinical score and paw thickness on days 28 and 35 post-immunization. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. FAST: fluorescent activatable sensor technology.

once the disease was comparatively severe. Interestingly, however, evaluation of inflammatory arthritis in CIA has recently been successfully performed using a fluorogenic MMP-3-specific polymeric probe [20], and an MMP-3-specific probe has been previously used to discriminate between CIA and naïve animals prior to clinically apparent disease [16]. MMP-12- and MMP-13-specific probes have been shown to correlate with CIA disease activity at early and late phases, respectively [36]. The variability of fluorescence obtained with MMPsense 750 in our hands may reflect a different sensitivity of the probe to different MMPs, and the role of MMPs in tissue

homeostasis and at different phases of disease. In an alternative model of arthritis, the anti-collagen antibody-induced model, MMPsense 680, has been shown to correlate with disease severity by being able to detect the disease-enhancing properties of circulating fibrocytes [37]. More recently, MMPsense 680 was successfully used to monitor OA development in mice, via direct injection of the dye through the patellar ligament into the knee joint cavity [19]. Demonstrating that MMP activity can be successfully assessed by near-infrared fluorescence imaging, depending on the experimental model and route of administration. The cathepsin K-specific probe, CatK

Fig. 3 Fluorescent signal from the neutrophil elastase-sensitive and the cathepsin-sensitive probe correlates with the histopathological score



(A) The mean histopathology score obtained from rear paws on day 35 in CIA mice treated with either the vehicle control (black) or dexamethasone (white). ** $P < 0.01$. (B) The correlation of the histological score with clinical score. (C) The correlation of the histological score with paw thickness. (D) The correlation of the Neutrophil Elastase 680 FAST rear paw FI with the histological score. (E) The correlation of the ProSense 750 FAST rear paw FI with the histological score. Correlation coefficient (r) and P -values are shown on the graphs. FAST: fluorescent activatable sensor technology.

FAST 680, also showed a lower degree of sensitivity, only showing increased fluorescence in animals with higher total clinical disease scores. This may also be reflective of cathepsin K's role in bone homeostasis and erosion [38] and it therefore being present at the highest levels only in the more severely affected animals.

Dexamethasone, a synthetic corticosteroid drug with substantial anti-inflammatory activities, has been shown previously to inhibit the development of experimental arthritis following immunization with type II collagen [39–41]. Reflecting the profound anti-arthritic effects of dexamethasone treatment in the CIA model, the fluorescence of the probes Neutrophil Elastase FAST 680 and ProSense FAST 750 were also reduced in treated mice. This is consistent with observations in other disease models where dexamethasone reduces pulmonary neutrophil elastase production [42] and in CIA (where another steroid drug prednisolone has been shown to reduce the numbers of neutrophils present within inflamed joints [43]).

The use of activatable probes and whole-body imaging for disease monitoring has a number of advantages over the standard methods. First, like clinical scoring, imaging can be used to assess the same animal over multiple time points—limited only by the half-life of the selected probes. However, unlike a simple clinical score, the activatable

probes provide information about the molecular processes occurring within tissues. The standard means of assessing the molecular processes of diseases in both animal models and clinically is assessment of serum titres of biomarkers [44, 45]. While repeated blood sampling is possible in animal models and in human patients, this may not fully reflect what is occurring at the site of pathology. In addition, where a mediator such as neutrophil elastase is localized on or in specific cells, this type of assessment allows the longitudinal monitoring of the infiltration and clearance of a specific cell population on an individual basis. An alternative method is to look for enzyme activity within joints [38]; however, this is more difficult in a clinical setting involving the biopsy or surgical removal of tissues and would usually be a terminal procedure in an animal model. Thus, repeated sampling from the same individual is not usually possible. Therefore, with the rapid development of new imaging probes, optical imaging may soon offer the possibility of assessing a broad range of molecular events in a non-invasive and multiplexed manner.

Most importantly, we have shown correlation between the probe FI and the histological score. Therefore, the proposed approach may decrease the number of animals required per experiment because tissue damage will not

necessarily require evaluation by harvesting joints for histology. The specificity of the different probes also provides information regarding the pathological processes occurring within the tissues. In preclinical studies, the imaging may also offer insight into disease in joints (such as the knees) that cannot usually be investigated by standard scoring techniques.

In conclusion, we have demonstrated that longitudinal non-invasive optical fluorescence imaging may represent a powerful tool for the assessment of murine CIA and efficacy of drugs. Optical imaging probes are beginning to make their way into the clinic. The relative ease and non-invasive nature of fluorescent molecular imaging techniques make them an attractive addition to disease assessment both in patients and in preclinical studies.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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