Pim Kinases as Therapeutic Targets in Early Rheumatoid Arthritis

Nicola J. Maney, Henrique Lemos, Ben Barron-Millar, Christopher Carey, Ian Herron, Amy E. Anderson, Andrew L. Mellor, John D. Isaacs, and Arthur G. Pratt

Objective. As well as being an established oncprotein and therapeutic target in cancer, proviral integration site for Moloney murine leukemia virus 1 (Pim-1) is implicated in human autoimmunity. This study was undertaken to investigate Pim-1 and its family members as potential therapeutic targets in early rheumatoid arthritis (RA).

Methods. A flow cytometry assay for PIM1 transcript measurement in peripheral blood mononuclear cells from patients with early arthritis was validated and applied as a biomarker of Pim-1 activity at the cellular level. Synovial protein expression was similarly determined by multiplex immunofluorescence in tissue samples from untreated RA patients and non-RA disease controls. Functional consequences of Pim kinase family manipulation in freshly isolated CD4+ T cells from these individuals were ascertained, along with the impact of Pim inhibition on mice with collagen-induced arthritis (CIA).

Results. The percentage of circulating CD4+ T cells positive for PIM1 transcript by flow cytometry proved a faithful surrogate for gene expression and was significantly higher in patients with early RA than in those with other diseases. Pim-1 protein levels were similarly up-regulated in synovial CD4+ T cells from patients with early RA. Ex vivo, exposure of T cell receptor–stimulated early RA CD4+ T cells to Pim kinase inhibitors restrained their activation and proliferative capacity. Diminished production of proinflammatory cytokines (interferon-γ and interleukin-17) and an expanded CD25highFoxP3+ Treg cell fraction were also observed in exposed versus unexposed cells. Finally, administration of Pim inhibitors robustly limited arthritis progression and cartilage destruction in CIA.

Conclusion. Our findings indicate that Pim kinases are plausible therapeutic targets in a readily identifiable subgroup of patients with early RA. Repurposing of Pim inhibitors for this disease should be considered.

INTRODUCTION

The proviral integration site for Moloney murine leukemia virus (Pim) family of oncoproteins comprises 3 constitutively active serine/threonine protein kinases, whose expression levels correlate with clinical outcome in a number of hematologic and solid tumors (1). Designated Pim-1, Pim-2, and Pim-3, their overlapping specificity for a range of substrates involved in cell survival, proliferation, and migration (2) can be explained by their amino acid sequence homology of >60% (2). Antiproliferative effects are mediated via activation of Bcl-2 antagonist of cell death (3), while phosphorylation of cyclin-dependent kinase inhibitor 1A (CDKN1A/p21Cip1/Waf1) (4) and CDKN1B/p27 (5) promotes cell cycle progression. While such observations have provided a rationale for the clinical development of Pim kinase inhibitors in oncology, recent findings suggest that the effectiveness of these drugs may also extend to nonmalignant diseases including skin psoriasis (6), inflammatory bowel disease (7), lupus nephritis (8), and rheumatoid arthritis (RA) (9). For example, alongside the antiproliferative and antimigratory effects that Pim inhibition exerts on synovial fibroblasts in RA, the approach also suppresses matrix metalloproteinase expression by these cells, with the potential to limit cartilage damage (9).

An increasingly apparent functional role for Pim kinases in shaping immune responses has yet to be exploited in...
immune-mediated inflammatory diseases. In particular, Pim-1 was shown to play a key part in the early stages of human Th1 differentiation (10), promoting interferon-γ (IFNγ) production while activating runt-related transcription factor, which in turn represses GATA-3 and hence Th2 differentiation (11). It has also been shown to inhibit the suppressive function of Treg cells (12). Such functions are expected to be enhanced during inflammation, since direct binding of pSTAT3 (13) or pSTAT5 (14) to the PIM1 promoter directly induces its transcription during cytokine signaling, and is further enhanced by NF-κB activation. Indeed, although variable in expression, PIM1 is strikingly up-regulated in circulating CD4+ T cells from untreated patients with early RA, differing significantly from that measured among disease controls even after correction for clinical parameters (15), a finding that we have independently validated and shown to be a consequence of sustained interleukin-6 (IL-6)-mediated STAT3 signaling (16,17).

Against this backdrop, we considered that Pim-1 inhibition might represent a viable therapeutic approach in early RA, which could be particularly effective in a readily identifiable subgroup of patients with high circulating CD4+ T cell PIM1 expression. This strategy might spare such individuals some of the “off-target” effects experienced with currently available modulators of up-stream IL-6/JAK/STAT signaling components, including Janus kinases and IL-6 receptor (18–20). Several small-molecule inhibitors that directly target the ATP binding domain of Pim-1 have already been developed for clinical use, all of which also inhibit Pim-2 and/or Pim-3 to a greater or lesser extent (21). For example, AZD1208 yielded acceptable tolerability data in phase I trials with ≤480 mg (22). An important consideration is whether analogous “pan-Pim” inhibitors that directly target the ATP binding domain of Pim-1 have independently validated and shown to be a consequence of sustained inhibitor directly induces its transcription during cytokine signaling, and is further enhanced by NF-κB activation. Indeed, although variable in expression, PIM1 is strikingly up-regulated in circulating CD4+ T cells from untreated patients with early RA, differing significantly from that measured among disease controls even after correction for clinical parameters (15), a finding that we have independently validated and shown to be a consequence of sustained interleukin-6 (IL-6)-mediated STAT3 signaling (16,17).

Against this backdrop, we considered that Pim-1 inhibition might represent a viable therapeutic approach in early RA, which could be particularly effective in a readily identifiable subgroup of patients with high circulating CD4+ T cell PIM1 expression. This strategy might spare such individuals some of the “off-target” effects experienced with currently available modulators of up-stream IL-6/JAK/STAT signaling components, including Janus kinases and IL-6 receptor (18–20). Several small-molecule inhibitors that directly target the ATP binding domain of Pim-1 have already been developed for clinical use, all of which also inhibit Pim-2 and/or Pim-3 to a greater or lesser extent (21). For example, AZD1208 yielded acceptable tolerability data in phase I trials with ≤480 mg (22). An important consideration is whether analogous “pan-Pim” inhibitors that directly target the ATP binding domain of Pim-1 have independently validated and shown to be a consequence of sustained interleukin-6 (IL-6)-mediated STAT3 signaling (16,17).

Against this backdrop, we considered that Pim-1 inhibition might represent a viable therapeutic approach in early RA, which could be particularly effective in a readily identifiable subgroup of patients with high circulating CD4+ T cell PIM1 expression. This strategy might spare such individuals some of the “off-target” effects experienced with currently available modulators of up-stream IL-6/JAK/STAT signaling components, including Janus kinases and IL-6 receptor (18–20). Several small-molecule inhibitors that directly target the ATP binding domain of Pim-1 have already been developed for clinical use, all of which also inhibit Pim-2 and/or Pim-3 to a greater or lesser extent (21). For example, AZD1208 yielded acceptable tolerability data in phase I trials with ≤480 mg (22). An important consideration is whether analogous “pan-Pim” inhibitors that directly target the ATP binding domain of Pim-1 have independently validated and shown to be a consequence of sustained interleukin-6 (IL-6)-mediated STAT3 signaling (16,17).

Against this backdrop, we considered that Pim-1 inhibition might represent a viable therapeutic approach in early RA, which could be particularly effective in a readily identifiable subgroup of patients with high circulating CD4+ T cell PIM1 expression. This strategy might spare such individuals some of the “off-target” effects experienced with currently available modulators of up-stream IL-6/JAK/STAT signaling components, including Janus kinases and IL-6 receptor (18–20). Several small-molecule inhibitors that directly target the ATP binding domain of Pim-1 have already been developed for clinical use, all of which also inhibit Pim-2 and/or Pim-3 to a greater or lesser extent (21). For example, AZD1208 yielded acceptable tolerability data in phase I trials with ≤480 mg (22). An important consideration is whether analogous “pan-Pim” inhibitors that directly target the ATP binding domain of Pim-1 have independently validated and shown to be a consequence of sustained interleukin-6 (IL-6)-mediated STAT3 signaling (16,17).

In vitro suppression of Pim kinase activity. For knock-down experiments, CD4+ T cells from healthy donors were isolated and stimulated, as described above, in the presence of 1 μM PIM1-specific small interfering RNA (siRNA) or nontargeting siRNA (SMARTpool siRNA; Dharmacon). The reduction in PIM1 mRNA was measured using real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR; see below). For protein inhibition experiments, CD4+ T cells were again isolated and stimulated as described above, this time in the presence of 20 μM (7.3 μg/ml) Pim-1 inhibitor (TCS PIM-1 1, also known as SC 204330; Tocris), 10 μM (3.8 μg/ml) pan-Pim inhibitor AZD1208 (BioVision), or an equivalent volume of DMSO as a control.

Real-time qRT-PCR. Total RNA was extracted from CD4+ T cells using an RNeasy Micro kit (Qiagen), according to the manufacturer’s instructions. Total RNA was reverse transcribed using SuperScript II reverse transcriptase and random hexamers, according to the manufacturer’s instructions (Invitrogen). PCR reactions were performed in duplicate using TaqMan Gene Expression Master Mix (Applied Biosystems) using primers and conditions described in the Supplementary Methods.

Flow cytometry. The antibodies used for flow cytometry are listed in Supplementary Table 1, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract. Standard flow cytometry procedures were carried out as described in the Supplementary Methods.

PrimeFlow RNA assay. A PrimeFlow RNA assay (ThermoFisher Scientific) was performed according to the manufacturer’s instructions. Briefly, after thawing and staining for viability
and surface markers, PBMCs were fixed for 30 minutes at 2–8°C, permeabilized with RNase inhibitors for 30 minutes at 2–8°C, and then further fixed for 1 hour at room temperature. The target/label probe sets used were PIM1 (Alexa Fluor 488, Type 4) and, as a positive control, RPL13A (Alexa Fluor 750, Type 6). Hybridization of the gene-specific oligonucleotide target probes to the target RNA sequence was performed for 2 hours at 40°C. Preamplifier and multiple amplifier molecules were then sequentially hybridized to target RNA for signal amplification (1.5 hours each at 40°C). Label probe oligonucleotides conjugated to fluorescent dyes were then added for 1 hour at 40°C before acquisition on a Fortessa X-20 flow cytometer. Data were analyzed using FlowJo software (Tree Star). The gating strategy for determining PIM1 transcript expression using this approach is depicted in Supplementary Figure 1, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract.

Tissue processing, quality control, and histologic analysis. Synovial tissue was paraffin-embedded using standard protocols between 24 and 72 hours after collection into 10% neutral buffered formalin. Four-micrometer hematoxylin and eosin–stained sections of sample blocks were considered valid for downstream analysis only if an intact cell lining layer was visible (Supplementary Figures 2A and B, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract). Subsequent staining was undertaken using automated DISCOVERY 5-plex Ventana immunohistochemistry technology (Roche Diagnostics), incorporating an antibody panel validated for this purpose (Supplementary Table 2, available on the website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract). With respect to the anti–Pim-1 antibody, appropriate staining in prostate tissue (where increased epithelial over stromal expression has been established in the literature [24]) was confirmed prior to use (Supplementary Figures 2C and D). For each synovial tissue section, a suitable field of view (FOV) was identified at 20× magnification and scanned using a Fortessa X-20 flow cytometer. Data were analyzed using FlowJo software (Tree Star). The gating strategy for determining PIM1 transcript expression using this approach is depicted in Supplementary Figure 1, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract.

Inform 2.4 software (PerkinElmer) was used to resolve unique spectra for individual fluorochromes and to analyze images. Cells in each individual case were segmented (based on nuclear staining/expected size), then phenotyped by 2-stage supervised machine learning. In the “training phase” 15–20 cells were manually phenotyped as CD3+CD4+ T cells, CD3+CD4− T cells, CD14+CD3− monocytes, or CD3−CD4−CD14− (other) cells, by a single observer who was blinded with regard to clinical data (NJM). In the subsequent “testing phase” the phenotype of all remaining cells in the FOV was predicted algorithmically (Supplementary Figure 3, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract).

Collagen-induced arthritis. All in vivo experiments were carried out according to the Animals (Scientific Procedures) Act 1986 in the Comparative Biology Centre of Newcastle University. Forty-five male DBA/1 mice (8–10 weeks old) were randomly allocated to separately ventilated cages (5 mice per labeled cage, each individually identifiable by ear notching and acclimated for 2 weeks. Animals were randomly assigned to 1 of 3 treatment groups before subcutaneous immunization at 2 sites at the base of the tail, each with a 100 μl emulsion containing 150 μg chicken type II collagen (Chondrex) dissolved in 50 μl 0.05M acetic acid and 50 μl Freund’s complete adjuvant (CFA; Sigma-Aldrich) containing 200 μg heat-killed Mycobacterium tuberculosis (BD Difco). On day 21, the same 100 μl emulsion was injected at 1 site, and on day 23, 25 μg of lipopolysaccharide from Escherichia coli (Chondrex) was injected intraperitoneally. Arthritis progression was monitored and scored as described in the Supplementary Methods. Upon onset of CIA, mice in this initial 70-day experiment were treated daily with either Pim-1 inhibitor (TCS PIM-1 1; 10 mg/kg/day by oral gavage) (n = 14 mice), a pan-Pim inhibitor (AZD1208; 30 mg/kg/day by oral gavage) (n = 15 mice), or vehicle alone (300 μl 5% DMSO, 5% Tween 20, 30% polyethylene glycol, 60% water; all volume/volume, by oral gavage) (n = 16 mice).

In a second experiment conducted in the same manner, 6 mice were treated with AZD1208 and 5 mice were treated with vehicle alone, and ankle joints were harvested and fixed in 10% neutral buffered formalin for 3 days at termination on day 41. Only paws inflamed at treatment initiation were harvested for histologic analysis. After decalcification and paraffin embedding, sections were stained with Weigert’s iron hematoxylin (incorporating an additional 0.5% acid alcohol wash), Safranin O (0.25%), and fast green (0.5%), as previously described (25). Cartilage destruction was scored on a 6-point semiquantitative scoring system (26) by 2 observers (HL and BB-M) who were blinded with regard to treatment group. No data points were excluded. All procedures were approved by the UK Home Office (Project license P1B4042BB).

Statistical analysis. Statistical procedures are described in the Supplementary Methods. Statistical analyses were conducted using GraphPad Prism software. Unless stated otherwise, P values less than 0.05 were considered significant.

RESULTS

PIM1 expression by T cells in early RA as a potential in vitro companion diagnostic. Having observed PIM1 gene expression to be up-regulated in circulating CD4+ T cells from untreated patients with early RA compared with disease controls (15,17), we sought a quantitative method for measurement of this parameter that could readily be applied in ex vivo material. A recently developed flow cytometry approach (PrimeFlow) enables relative RNA quantitation at the single-cell level, and was evaluated for this purpose. To validate this assay, PrimeFlow readouts
in CD4+ T cells within PBMCs were compared with normalized PIM1 expression in purified CD4+ T cells obtained at the same blood draw, measured by the gold standard TaqMan qRT-PCR assay. This comparison confirmed PrimeFlow measurement as a faithful surrogate for gene expression at the cellular level, with strong correlation between readouts ($P = 0.0022$) (Figure 1A).

PIM1 expression was then systematically ascertained using a PrimeFlow assay in a newly recruited cohort of untreated patients with inflammatory arthritis. Characteristics of the RA and disease control groups are presented in Table 1. The groups were matched for markers of systemic inflammation (C-reactive protein [CRP] and erythrocyte sedimentation rate [ESR]) and were representative of NEAC patients more generally (15–17). Somewhat increased PIM1 gene expression was observed in PBMCs from patients with early RA compared with patient controls with other diseases ($P = 0.011$) (Figure 1B). This was in large part accounted for by strikingly increased PIM1 expression in circulating T cells ($P = 0.003$) (data not shown), and specifically the CD4+ T cell compartment ($P = 0.004$) (Figure 1C); interestingly, the observation was mirrored in the CD4− T cell subpopulation presumed to comprise predominantly CD8+ T cells ($P = 0.002$) (Figure 1D). In contrast, no significant difference between patient groups was identifiable in circulating monocytes (Figure 1E).

IL-6 drives CRP production as well as STAT3 signaling upstream of PIM1 expression, and an association between CRP and T cell transcript was observed in the circulating CD4+ (though not CD4−) cell subsets in our cohort, where paired data were available (Supplementary Figures 5A–F, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract). This finding is consistent with prior observations in larger cohorts, where increased CD4+ T cell PIM1 expression in patients with early RA has nonetheless been shown to be independent of acute-phase response (16). Our data cannot fully exclude confounding effects of relative CD4+ T

Figure 1. Flow cytometric assessment of PIM1 RNA expression in T cells using a PrimeFlow RNA assay. A, Correlation between PIM1 gene expression assessed by PrimeFlow in CD4+ T cells within peripheral blood mononuclear cells (PBMCs) from patients with early rheumatoid arthritis (RA) and non-RA disease controls, and PIM1 gene expression assessed by quantitative reverse transcriptase–polymerase chain reaction in freshly isolated CD4+ T cells obtained at the same blood draw as PBMCs. Samples were analyzed after 1 freeze–thaw cycle. Pearson's correlation coefficient ($R^2$) is shown. B–E, PrimeFlow measurement of PIM1 expression in total PBMCs (B), CD4+ T cells (C), CD4− T cells (presumed to be predominantly CD8+ cells) (D), and CD14+ monocytes (E) from RA patients and non-RA disease controls. Symbols represent individual patients; bars show the mean ± SEM. * = $P \leq 0.05$; ** = $P \leq 0.01$, by Mann-Whitney U test. NS = not significant.
MANEY ET AL

1824

| Cell subset compositions as an explanation for differential PIM1 expression between disease phenotypes; however, based on reanalysis of previously published peripheral blood flow cytometry data from the NEAC cohort, discrepancies in naive versus memory cell frequencies between comparator groups are unlikely to account for transcriptional up-regulation in early RA (16, 27) (Supplementary Figures 5G–I). Taken together, these observations pinpoint T cell–specific PIM1 gene expression measurement as a tractable and potentially reliable stratification tool in early RA.

Increased Pim-1 protein levels among infiltrating CD4+ T cells in early RA synovium. To further evaluate the relevance of Pim-1 during the early stages of RA, we examined protein expression in synovial tissue prior to commencement of immunomodulatory therapy. We hypothesized that enhanced Pim-1 protein expression by infiltrating T cells, and specifically CD4+ T cells, in the synovium might further define the disease. A multiplex immunofluorescence approach was adopted. A total of 25 synovial tissue samples of suitable quality were available from patients with untreated inflammatory arthritis, whose clinical and demographic characteristics are summarized in Table 2.

Figure 2A shows staining of a representative tissue section, with Pim-1–positive and Pim-1–negative CD4+ T cells indicated. The relative proportions of synovial tissue cell subsets characterized were similar among RA patients and disease controls (Supplementary Figure 6A, Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract), with Pim-1–positive and Pim-1–negative CD4+ T cells indicated.

Table 1. Characteristics of the untreated patients with rheumatoid arthritis (RA) and non-RA control subjects included in the PrimeFlow analysis of peripheral blood*

|                  | RA (n = 21) | Non-RA (n = 14)† | P‡ |
|------------------|------------|------------------|----|
| Age, years       | 63 (44–84) | 46 (28–88)       | 0.012 |
| Sex, % female    | 57         | 57               | NS  |
| No. of tender joints (74 assessed) | 12 (0–50) | 3.5 (0–41) | NS  |
| No. of swollen joints (72 assessed) | 3 (1–23) | 2.5 (0–13) | NS  |
| CRP, gm/liter    | 22 (<5–96) | 8 (<5–160)       | NS  |
| ESR, mm/hour     | 29.5 (2–82) | 18.5 (2–90)     | NS  |
| RF positive, %   | 67         | 14               | 0.007 |
| ACPA positive, % | 57         | 0                | 0.002 |
| DAS28- CRP       | 4.52 (2.24–7.47) | – | – |
| DAS28- ESR       | 4.84 (2.06–7.59) | – | – |

* Except where indicated otherwise, values are the median (range). NS = not significant; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; RF = rheumatoid factor; ACPA = anti–citrullinated protein antibody; DAS28-CRP = Disease Activity Score in 28 joints using the CRP level.
† Non-RA diagnoses included psoriatic arthritis in 5 patients (36%), other spondyloarthritis in 4 patients (29%), systemic lupus erythematosus in 1 patient (7%), gout in 1 patient (7%), and other in 3 patients (21%). Other non-RA inflammatory arthritides included 2 cases of self-limiting undifferentiated inflammatory arthritis and 1 case of streptococcal-associated reactive arthritis.
‡ By Mann-Whitney U test for continuous variables; by chi-square test for dichotomous variables.

Table 2. Characteristics of the untreated patients with RA and non-RA control subjects included in the synovial tissue analysis*

|                  | RA (n = 16) | Non-RA (n = 9)† | P‡ |
|------------------|------------|------------------|----|
| Age, years       | 61 (41–85) | 63 (44–72)       | NS  |
| Sex, % female    | 45         | 67               | 0.002 |
| Wrist biopsied, %| 88         | 78               | NS  |
| No. of tender joints (74 assessed) | 6 (0–35) | 4 (1–18) | NS  |
| No. of swollen joints (72 assessed) | 4 (2–23) | 4 (1–9) | NS  |
| CRP, gm/liter    | 22.5 (<5–78) | 12.5 (5–171) | NS  |
| ESR, mm/hour     | 28 (2–76) | 30 (1–126)       | NS  |
| RF positive, %   | 50         | 22               | <0.001 |
| ACPA positive, % | 56         | 0                | <0.001 |
| DAS28- CRP       | 4.34 (2.37–7.47) | 4.1 (2.35–5.87) | NS  |
| DAS28- ESR       | 4.53 (1.54–7.43) | 6.57 (0.74–6.12) | NS  |

* Except where indicated otherwise, values are the median (range). See Table 1 for definitions.
† Non-RA diagnoses included psoriatic arthritis in 4 patients (44%), other spondyloarthritis in 2 patients (22%), gout in 2 patients (22%), and other in 1 patient (12%).
‡ By Mann-Whitney U test for continuous variables; by chi-square test for dichotomous variables.
§ Knee synovium was biopsied in the remainder of the patients.
but Pim-1 protein was expressed at higher levels in cells from patients with early RA compared with disease controls (Figure 2B). Further quantitative analysis indicated that increased expression among infiltrating T cells accounted for this discrepancy, with a contribution from both CD4+ and CD4− subpopulations (Figures 2C and D). In contrast, no significant difference in Pim-1 expression among CD14+ CD3− myeloid cells was seen between comparator groups (Figure 2E). Finally, we investigated whether there was a relationship between synovial tissue Pim-1 protein expression and PBMC PIM1 gene expression (Supplementary Figures 6B and C); any potential association did not reach statistical significance in this small sample set (Supplementary Figure 6B).

Decreased inflammatory effector function of early RA CD4+ T cells cultured with Pim inhibitors. Our observations suggested that up-regulation of Pim-1 in circulating and synovial CD4+ T cells is a feature of early disease in RA patients. The previously described proinflammatory role of Pim kinases in adaptive immunity (10–12,28–30), combined with their ability to drive synovial fibroblast proliferation (10), fuels interest in them as therapeutic targets for RA. We therefore sought to confirm the consequences of disrupted Pim kinase function in primary CD4+ T cells, and the extent to which these effects could be mediated by Pim-1–selective inhibition versus pan-Pim inhibition in early RA, reasoning that our findings might inform selection of the optimal therapeutic strategy for development.
First, 41% knockdown was achieved with PIM1-specific siRNA relative to nontargeting control siRNA (Supplementary Figure 7A, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract), and significantly reduced activation and proliferation of CD4+ T cells from healthy donors was evident 3 days after stimulation with anti-CD3/anti-CD28 (Supplementary Figures 7B and C). Focusing on CD4+ T cells freshly isolated from blood from untreated patients with early RA, we then explored the impact on effector function of commercially available small molecule inhibitors that either selectively target Pim-1 (TCS-PIM-1 1) or target all 3 Pim kinases (AZD1208). After 3 days of CD4+ T cell stimulation, both inhibitors significantly decreased activation (CD25 expression) and proliferation as determined by CellTrace Violet staining, with no significant impact on cell viability (Supplementary Figures 7D–F). This effect was sustained in each case at 6 days (Figures 3A and B), again with minimal diminution in cell viability (Supplementary Figure 7G).

Production of the proinflammatory cytokine IFNγ by cells treated with either inhibitor was significantly reduced following restimulation at day 6, and this result was also seen for IL-17 (only significant following Pim-1–specific inhibition) (Figures 3C and D and Supplementary Figures 8A–D, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41744/abstract). In contrast, we observed for the first time that Pim inhibition led to significantly enhanced expansion of regulatory CD4+ T cells (Treg cells); indeed, after pan-Pim (but not Pim-1–specific) inhibition, a 2-fold increased frequency of CD25highFoxP3+ Treg cells was observed (Figures 3E and F and Supplementary Figures 8E–H). Taken together, these data indicate that the proinflammatory effector function of CD4+ T cells from patients with early RA is restrained by Pim kinase inhibition.

![Figure 3](image_url)
Amelioration of CIA by both Pim-1–selective inhibition and pan-Pim inhibition. Our current and previous data implicate STAT3 signaling in CD4+ T cells as an early event in RA pathogenesis that may result in aberrant proinflammatory effector responses via overexpression of PIM1 (15,17,27). Considered alongside complementary data recently published in relation to stromal cells (9), we therefore reasoned that applying pharmacologic Pim kinase inhibition to a model of inflammatory arthritis might abrogate disease severity. In one such model, CIA, a destructive symmetrical polyarthritis resembling RA develops in the DBA/1 mouse following immunization with type II collagen in CFA.

Since our experiments using primary CD4+ T cells from patients with early RA suggested that Pim-1–selective and pan-Pim kinase inhibition had comparable effects on effector function, we compared the therapeutic impact of both approaches in CIA using TCS-PIM-1 1 and AZD1208, respectively. Mice developed CIA after a median of 24 days, and were treated at clinical onset with either drug or vehicle alone by daily oral gavage for a total of 25 days (until day 49). Clinical features were evaluated...
longitudinally until day 70. A clear reduction in the clinical severity of arthritis in mice treated with TCS-PIM-1 was seen, which became significant after 31 days. Arthritis scores increased after treatment cessation, beginning to approach those seen in the control arm by the end of the experiment (Figure 4A). A similar pattern of CIA amelioration was seen in mice treated with AZD1208, with a somewhat larger and more sustained effect apparent (Figure 4B). In a separate experiment, pan-Pim inhibition markedly reduced cartilage destruction after 40 days of treatment (Figures 4C–E). These results indicate that Pim kinase inhibition, whether specific for Pim-1 or not, significantly abrogates the progression of arthritis in a model that resembles RA, and cartilage destruction is significantly reduced by administration of this inhibitor class.

**DISCUSSION**

A growing array of available therapeutic options, the recognized importance of prompt diagnosis, and widespread adoption of “tight control” management strategies have together transformed clinical outcomes for RA patients in recent years. Nonetheless, remission rates remain disappointingly low at 20–30%, even among patients with recently diagnosed disease (31). In another 20%, the disease is refractory to multiple available treatments (32), and RA continues to be associated with impaired quality of life, disability, and work instability (33). Efforts to address these unmet needs are hampered by an inability to identify the optimal treatment for each patient, based on relevant pathophysiology. The present study is notable in this context, highlighting repurposing of Pim kinase inhibition as a rational therapeutic strategy for a subgroup of patients with early RA that is potentially identifiable by a companion molecular assay. Interventional clinical studies to test this hypothesis are awaited and, in our view, warranted to build on convergent preclinical data.

A persuasive body of evidence for the involvement of Pim signaling in RA pathogenesis is now apparent. As transcriptionally regulated kinases that are sensitive to JAK/STAT signaling (via promoter sequences that directly bind activated STAT3 and STAT5) (13,14), Pim induction is an anticipated consequence of synovial fibroblast (RASF) homeostasis further supports its candidacy as a therapeutic target (9). Dysregulation of cyclin-dependent kinase pathways is well recognized in tumor biology—explaining the interest in Pim kinase inhibition for malignancies (37)—but has also been reported in RASFs (38,39), leading to the hypothesis that these cells contribute to and maintain synovitis and account for the apparent “ceiling effect” of established therapies that exclusively target immune cells and cytokines. Hence, while an improved understanding of Pim kinases in CD4+ T cell–mediated RA induction was the emphasis of the present work, concurrent targeting of stromal pathobiology potentially represents an additional beneficial effect of Pim kinase inhibition in RA. Encouraging results from our in vivo experiments provide valuable proof-of-concept for this approach and a platform for clinical studies.

Previous work by our group has consistently shown PIM1 gene expression in CD4+ T cells freshly isolated from peripheral blood to be significantly elevated during the development of RA compared with other diseases in an early arthritis clinic, being a component of a STAT3-regulated transcriptional program in these cells (15–17). Validation of a flow cytometry assay for PIM1 transcript measurement at a cellular level was undertaken in the absence of specific antibodies suitable for conventional flow cytometry (40). The assay circumvents the need to isolate leukocyte subsets, and validation of the technique against gold standard qRT-PCR readouts introduces a method that holds promise as a tractable test, using frozen PBMC samples. Our data further suggest that the potential value of peripheral blood as a surrogate of synovial Pim-1 expression deserves further study, potentially increasing the value of testing as a potential companion diagnostic to a matched therapy. Further validation work will be required to confirm these properties and, in particular, the test’s potential to predict the efficacy of Pim kinase inhibition in RA. Its appraisal for this purpose will be possible only within the setting of a clinical trial.

In the present study, the comparative merits of Pim-1–specific inhibition versus pan-Pim inhibition were considered. In vitro studies suggested that both approaches had similar effects in restraining CD4+ T cell activation, proliferation, and Th1 differentiation. More convincing Treg cell induction (but less convincing restraint of Th17 differentiation) was observed using the pan-Pim strategy. Reduced circulating frequencies and impaired function of Treg cells in early RA, as well as restoration of function following successful therapy under certain circumstances, have been reported (41,42), and Treg cell expansion continues to be actively pursued as a therapeutic strategy in autoimmunity (43). In contrast, therapeutic targeting of the IL-23/IL-17 axis in
PIM KINASES AS THERAPEUTIC TARGETS IN EARLY RA

RA has yielded disappointing results in the clinic (44,45). Conceivably, the comparatively sustained repression of CIA even after withdrawal of pan-Pim inhibition (Figures 4A and B) may reflect the more potent Treg cell induction observed in vitro using this approach (Figure 3F). We therefore propose that nonspecific inhibition will be the optimal approach for advancement in RA. Indeed, the suggestion that Pim-1-specific blockade may lead to compensatory up-regulation of other kinase family members with strongly overlapping biologic effects—and hence the potential to dampen therapeutic responses—would support this strategy (46,47).

The AZD1208 compound used as a pan-Pim inhibitor in the present investigation was shown to be a potent inducer of cytchrome P450 3A4 (CYP3A4) after multiple dosing in recent early phase clinical trials, leading to accelerated drug clearance and unfavorable pharmacodynamics that precluded ongoing development, despite evidence of biologic activity (22). This issue has not been reported for alternative agents, of greater potency, development, despite evidence of biologic activity (22). This issue and unfavorable pharmacodynamics that precluded ongoing early phase clinical trials, leading to accelerated drug clearance in the present investigation was shown to be a potent inducer of cell motility. Int J Biochem Cell Biol 2017;49:728–40.

The authors gratefully acknowledge the participation of all patients and healthy volunteers. The authors also thank Ben Hargreaves (database manager) for administrative support and Imogen Wilson for technical support. The authors gratefully acknowledge Claire Jones (MRC Molecular Pathology Node, Newcastle upon Tyne Hospitals NHS Foundation Trust) for expert support.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the participation of all patients and healthy volunteers. The authors also thank Ben Hargreaves (database manager) for administrative support and Imogen Wilson for technical support. The authors gratefully acknowledge Claire Jones (MRC Molecular Pathology Node, Newcastle upon Tyne Hospitals NHS Foundation Trust) for expert support.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Pratt had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Maney, Lemos, Barron-Millar, Anderson, Mellor, Pratt.

Acquisition of data. Maney, Lemos, Barron-Millar, Carey, Herron.

Analysis and interpretation of data. Maney, Lemos, Barron-Millar, Carey, Herron, Anderson, Mellor, Isaacs, Pratt.

REFERENCES

1. Santio NM, Koskinen PJ. PIM kinases: from survival factors to regulators of cell motility. Int J Biochem Cell Biol 2017;49:728–40.

2. The UniProt Consortium. UniProt: the universal protein knowledgebase. Nucleic Acids Res 2017;45:D158–69.

3. Macdonald A, Campbell DG, Toth R, Mc Laughlan H, Hastie CJ, Arthur JS. Pim kinases phosphorylate multiple sites on Bad and promote 14-3-3 binding and dissociation from Bcl-XL. BMC Cell Biol 2006;7:1.

4. Zhang Y, Wang Z, Magnuson NS. Pim-1 kinase-dependent phosphorylation of p21Cip1/WAF1 regulates its stability and cellular localization in H1299 cells. Mol Cancer Res 2007;5:909–22.

5. Morishita D, Katayama R, Sekimizu K, Tsuruo T, Fujita N. Pim kinases promote cell cycle progression by phosphorylating and down-regulating p27Kip1 at the transcriptional and posttranscriptional levels. Cancer Res 2008;68:5076–85.

6. Perera GK, Ainali C, Semenova E, Hundhausen C, Barinaga G, Kassen D, et al. Integrative biology approach identifies cytokine targeting strategies for psoriasis. Sci Transl Med 2014;6:223ra22.

7. Jackson LJ, Pheneger JA, Pheneger TJ, Davis G, Wright AD, Robinson JE, et al. The role of PIM kinases in human and mouse CD4+ T cell activation and inflammatory bowel disease. Cell Immunol 2012;272:200–13.

8. Fu R, Xia Y, Li M, Mao R, Guo C, Zhou M, et al. Pim-1 as a therapeutic target in lupus nephritis. Arthritis Rheumatol 2019;71:1308–18.

9. Ha YJ, Choi YS, Han DW, Kang EH, Yoo IS, Kim JH, et al. PIM-1 kinase is a novel regulator of proinflammatory cytokine-mediated responses in rheumatoid arthritis fibroblast-like synoviocytes. Rheumatology (Oxford) 2019;58:154–64.

10. Aho TL, Lund RJ, Ylikoski EK, Matikainen S, Lahesmaa R, Koskinen PJ, et al. Expression of human Pim family genes is selectively up-regulated by cytokines promoting T helper type 1, but not T helper type 2, cell differentiation. Immunology 2005;116:82–8.

11. Tahvanainen J, Kylanieni MK, Kanduri K, Gupta B, Lahteenmaki H, Kaltonen T, et al. Proviral integration site for Moloney murine leukemia virus (PIM) kinases promote human T helper cells differentiation. J Biol Chem 2013;288:3048–58.

12. Li Z, Lin F, Zhuo C, Deng G, Chen Z, Yin S, et al. PIM1 kinase phosphorylates the human transcription factor FOXP3 at serine 422 to negatively regulate its activity under inflammation. J Biol Chem 2014;289:26872–81.

13. Shirogane T, Fukada T, Muller JM, Shima DT, Hibi M, Hirano T. Synergistic roles for Pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis. Immunity 1999;11:709–19.

14. Nosioka T, Kawashima T, Misawa K, Ikuta K, Mui AL, Kitamura T. STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. EMBO J 1999;18:4754–65.

15. Pratt AG, Swan DC, Richardson S, Wilson G, Hilkens CM, Young DA, et al. A CD4 T cell gene signature for early rheumatoid arthritis implicates interleukin 6-mediated STAT3 signalling, particularly in anti-citrullinated peptide antibody-negative disease. Ann Rheum Dis 2012;71:1374–81.

16. Andere AE, Pratt AG, Sedhom MA, Doran JP, Routledge C, Hargreaves B, et al. IL-6-driven STAT signalling in circulating CD4+ lymphocytes is a marker for early anticitrullinated peptide antibody-negative rheumatoid arthritis. Ann Rheum Dis 2018;75:466–73.

17. Andere AE, Maney NJ, Nair N, Lendrem DW, Skalton AJ, Diboll J, et al. Expression of STAT3-regulated genes in circulating CD4+ T cells discriminates rheumatoid arthritis independently of clinical parameters in early arthritis. Rheumatology (Oxford) 2019;58:1250–8.

18. Bechman K, Subasinghe S, Norton S, Atzeni F, Galli M, Cope AP, et al. A systematic review and meta-analysis of infection risk with
small molecule JAK inhibitors in rheumatoid arthritis. Rheumatology (Oxford) 2019;58:1756–66.

19. Pawar A, Desai RJ, Solomon DH, Ortiz AJ, Gale S, Bao M, et al. Risk of serious infections in tocilizumab versus other biologic drugs in patients with rheumatoid arthritis: a multidatabase cohort study. Ann Rheum Dis 2019;78:456–64.

20. Xi F, Yun H, Bernatsky S, Curtis JR. Risk of gastrointestinal perforation among rheumatoid arthritis patients receiving tofacitinib, tocilizumab, or other biologic treatments. Arthritis Rheumatol 2016;68:2612–7.

21. Zhang X, Song M, Kundu JK, Lee MH, Liu ZZ. PIM kinase as an executional target in cancer. J Cancer Prev 2018;23:109–16.

22. Cortes J, Tamura K, DeAngelo DJ, de Bono J, Lorente D, Minden M, et al. Phase I studies of AZD1208, a proviral integration Moloney virus kinase inhibitor in solid and haematological cancers. Br J Cancer 2018;118:1425–33.

23. Kelly S, Humby F, Filer A, Ng N, Di Cicco M, Hands RE, et al. Ultrasound-guided synovial biopsy: a safe, well-tolerated and reliable technique for obtaining high-quality synovial tissue from both large and small joints in early arthritis patients. Ann Rheum Dis 2015;74:611–7.

24. Gibull TL, Jones TD, Li L, Eble JN, Baldridge LA, Malott SR, et al. Over-expression of Pim-1 during progression of prostate carcinoma. J Clin Pathol 2006;59:285–8.

25. Schmitz N, Laverty S, Kraus VB, Aigner T. Basic methods in histopathology of joint tissues. Osteoarthritis Cartilage 2010;18 Suppl 3:S113–6.

26. Glasson SS, Chambers MG, van den Berg WB, Little CB. The OARSI histopathology initiative—recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis Cartilage 2010;18 Suppl 3:S17–23.

27. Ridgley LA, Anderson AE, Maney NJ, Naamane N, Skelton AJ, Lawson CA, et al. IL-6 mediated transcriptional programming of naive CD4+ T cells in early rheumatoid arthritis drives dysregulated effector function. Front Immunol 2019;10:1535.

28. Jacobs H, Krimpenfort P, Haks M, Allen J, Blom B, Demolliere C, et al. PIM1 reconstitutes thymus cellularity in interleukin 7- and common γ-chain-mutant mice and permits thymocyte maturation in Rag-but not CD3γ-deficient mice. J Exp Med 1999;190:1059–68.

29. Schmidt T, Karsunky H, Mathieu N, Schmidt T, Verthuy C, Ferrier P, et al. The Pim-1 kinase stimulates maturation of TCRβ-deficient T cell progenitors: implications for the mechanism of Pim-1 action. Int Immunol 2000;12:1389–96.

30. Jacobs H, Krimpenfort P, Haks M, Allen J, Blom B, Demolliere C, et al. PIM1 reconstitutes thymus cellularity in interleukin 7- and common γ-chain-mutant mice and permits thymocyte maturation in Rag-but not CD3γ-deficient mice. J Exp Med 1999;190:1059–68.

31. Leduc I, Karsunky H, Mathieu N, Schmidt T, Verthuy C, Ferrier P, et al. The Pim-1 kinase stimulates maturation of TCRβ-deficient T cell progenitors: implications for the mechanism of Pim-1 action. Int Immunol 2000;12:1389–96.

32. Schmidt T, Karsunky H, Rodel B, Zevnik B, Elasser HP, Moroy T, Evidence implicating Gfi-1 and Pim-1 in pre-T-cell differentiation steps associated with beta-selection. EMBO J 1998;17:5349–59.

33. Yu C, Jin S, Wang Y, Jiang N, Wu C, Wang Q, et al. Remission rate and predictors of remission in patients with rheumatoid arthritis under treat-to-target strategy in real-world studies: a systematic review and meta-analysis. Clin Rheumatol 2019;38:727–38.

34. Buch MH. Defining refractory rheumatoid arthritis. Ann Rheum Dis 2018;77:966–9.

35. Safii S, Kolah AA, Hoy D, Smith E, Bettampadi D, Mansourina MA, et al. Global, regional and national burden of rheumatoid arthritis 1990–2017: a systematic analysis of the Global Burden of Disease study 2017. Ann Rheum Dis 2019;78:1463–71.

36. Brautl L, Gasser C, Bracher F, Huber K, Knapp S, Schwaller J. PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers [review]. Haematologica 2010;95:1004–15.

37. Blanco-Aparicio C, Camero A. Pim kinases in cancer: diagnostic, prognostic and treatment opportunities. Biochem Pharmacol 2013;85:629–43.

38. Nasu K, Kohsaka H, Nonomura Y, Terada Y, Ito H, Hirokawa K, et al. Adenoviral transfer of cyclin-dependent kinase inhibitor genes suppresses collagen-induced arthritis in mice. J Immunol 2000;165:7246–52.

39. Perlman H, Bradley K, Liu S, Cole S, Shamiyeh E, Smith RC, et al. IL-6 and matrix metalloproteinase-1 are regulated by the cyclin-dependent kinase inhibitor p21 in synovial fibroblasts. J Immunol 2003;170:838–45.

40. Depreter B, Philippé J, Meul M, Denys B, Vandepoele K, De Moerloose B, et al. Cancer-related mRNA expression analysis using a novel flow cytometry-based assay [review]. Cytometry B Clin Cytom 2018;94:565–75.

41. Ehrenstein MR, Evans JG, Singh A, Moore S, Wames G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. J Exp Med 2004;200:277–85.

42. Lawson CA, Brown AK, Bejarano V, Douglas SH, Burgoyne CH, Greenstein AS, et al. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25+regulatory T cell population in peripheral blood. Rheumatology (Oxford) 2006;45:1210–7.

43. Rosenzwaig M, Lorenzon R, Cacoub P, Pham HP, Pitoiset F, El Soufi K, et al. Immunological and clinical effects of low-dose interleukin-2 across 11 autoimmune diseases in a single, open clinical trial. Ann Rheum Dis 2019;78:209–17.

44. Bianco FJ, Moricke R, Dokoupilova E, Coddin C, Neal J, Anderson M, et al. Secukinumab in active rheumatoid arthritis: a phase III randomised, double-blind, active comparator- and placebo-controlled study. Arthritis Rheumatol 2017;69:1144–53.

45. Smolen JS, Agarwal SK, Ilivanoa E, Xu XL, Miao Y, Zhang Y, et al. A randomised phase II study evaluating the efficacy and safety of subcutaneously administered ustekinumab and guselkumab in patients with active rheumatoid arthritis despite treatment with methotrexate. Ann Rheum Dis 2017;76:831–9.

46. Garcia PD, Langowski JL, Wang Y, Chen M, Castillo J, Fanton C, et al. Pan-PIM kinase inhibition provides a novel therapy for treating hematologic cancers. Clin Cancer Res 2014;20:1834–45.

47. Koblish H, Li YL, Shin N, Hall L, Wang G, Wang K, et al. Preclinical characterization of INC053914, a novel pan-PIM kinase inhibitor, alone and in combination with anticancer agents, in models of hematologic malignancies. PLoS One 2018;13:e0199108.

48. Chen Q, Wang Y, Shi S, Li K, Zhang L, Gao J. Insights into the interaction mechanisms of the proviral integration site of moloney murine leukemia virus (Pim) kinases with pan-Pim inhibitors PIM447 and AZD1208: a molecular dynamics simulation and MM/GBSA calculation study. Int J Mol Sci 2019;20:5410.