HIF1α inhibition facilitates Leflunomide-AHR-CRP signaling to attenuate bone erosion in CRP-aberrant rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by progressive bone erosion. Leflunomide is originally developed to suppress inflammation via its metabolite A77 1726 to attenuate bone erosion. However, distinctive responsiveness to Leflunomide is observed among RA individuals. Here we show that Leflunomide exerts immunosuppression but limited efficacy in RA individuals distinguished by higher serum C-reactive protein (CRP Henderson, CRP Henderson), whereas the others with satisfactory responsiveness to Leflunomide show lower CRP (CRP Lower, CRP Lower). CRP inhibition decreases bone erosion in arthritic rats. Besides the immunomodulation via A77 1726, Leflunomide itself induces AHR-ARNT interaction to inhibit hepatic CRP production and attenuate bone erosion in CRP Lower arthritic rats. Nevertheless, high CRP in CRP Henderson rats upregulates HIF1α, which competes with AHR for ARNT association and interferes Leflunomide-AHR-CRP signaling. Hepatocyte-specific HIF1α deletion or a HIF1α inhibitor Acriflavine re-activates Leflunomide-AHR-CRP signaling to inhibit bone erosion. This study presents a precision medicine-based therapeutic strategy for RA.
Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammatory synovial hyperplasia, stiffness, progressive bone erosion and systemic features. Progressive bone erosion is a prominent determinant of functional impairment of joints in RA. Currently, a range of treatment options including conventional and biologic antirheumatic drugs are available. However, none of them is universally effective. Understanding the mechanisms underlying the non-responses of these drugs will provide new insight into development of precision medicine-based therapeutic strategy for RA.

During RA development, multiple immune cells and cytokines are organized within a hierarchical regulatory network that favors osteoclast-mediated bone erosion. Leflunomide has been widely used in RA treatment and the well-known mechanism is immunomodulation via its metabolite A77 1726. Briefly, Leflunomide is converted to A77 1726 in intestinal wall and liver after oral administration. A77 1726 inhibits mitochondrial dihydroorotate dehydrogenase (DHODH) required for proliferation of immune cells, resulting in attenuation of inflammation-induced bone erosion. However, only 40–50% RA patients receiving Leflunomide meet 20% reduction in disease activity according to American College of Rheumatology response criteria.

In this study, we classified RA patients treated with Leflunomide based on presence of progressive bone erosion (PBE-positive, PBE+) or not (PBE-negative, PBE−). Interestingly, Leflunomide significantly inhibited inflammation in both PBE− and PBE+ patients, suggesting the limited efficacy of Leflunomide in attenuating bone erosion in PBE+ patients was not coupled with its immunomodulatory action. The PBE− patients had higher serum C-reactive protein (CRPHigher, CRPH), whereas the PBE− patients showed relatively lower serum CRP (CRPlow, CRPL). Studies have demonstrated that CRP of hepatic origin is involved in osteoclastogenesis.

In summary, this study reveals that CRP-HIF1α signaling axis is responsible for the limited efficacy of Leflunomide in CRPH RA. On the basis of this finding, we develop a precision medicine-based therapeutic strategy for CRPH RA, i.e., the combination of Leflunomide and ACF.

**Results**

**Limited efficacy of Leflunomide in CRPH RA patients.** We reviewed radiographic data of 250 RA patients treated with Leflunomide (Supplementary Table 1). Leflunomide significantly attenuated progressive bone erosion in 130 RA patients (PBE−) but showed limited efficacy in the rest 120 RA patients (PBE+) (Fig. 1a, b). However, inhibition of DHODH activity and proliferation of immune cells (T and B lymphocytes and macrophages) were comparable between PBE− and PBE+ patients. Cytokines produced by immune cells and inflammatory synovial fibroblasts including Interleukin-17 (IL-17), Interleukin-6 (IL-6), and receptor activator of nuclear factor kappa-B ligand (RANKL) also showed no difference between the two RA groups (Fig. 1c and Supplementary Fig. 1a). We determined the associations between PBE+ patients and serum baseline blood indicators including rheumatoid factors (IGM, IGG and IGA), CRP, anti-cyclic citrullinated peptide (anti-CCP) antibody and erythrocyte sedimentation rate (ESR) (Fig. 1d). Serum CRP showed high specificity and sensitivity for PBE+ RA patients and the diagnostic accuracy was above 92% (Fig. 1d, Supplementary Fig. 1b and Supplementary Table 2). The PBE− patients demonstrated higher levels of serum baseline CRP (CRP1) and a bone resorption marker (tartrate-resistant acid phosphatase 5b, TRAP5b) (Fig. 1e). Serum CRP, rather than other indicators, was positively associated with TRAP5b in CRPH RA patients (Fig. 1f) and Supplementary Fig. 1c). Role of CRP in osteoclastogenesis are conformation- and RANKL-dependent. Circulating native CRP is composed of five identical subunits and dissociates into the monomeric conformation upon entering local lesions. Monomeric CRP promotes osteoclast differentiation in the absence of RANKL but inhibits RANKL-induced osteoclast differentiation by neutralizing RANKL.

**Limited efficacy of Leflunomide in CRPH CIA rats.** We established CIA rat models and administered the CIA rats with Leflunomide for 28 days (Supplementary Fig. 2a). The CIA rats were also classified into PBE− and PBE− subgroups based on microCT analysis (Supplementary Fig. 2a). We observed the progressively deteriorated bone microarchitecture and decreased bone mass in PBE+ rats rather than in PBE− rats after Leflunomide treatment (Fig. 2a and Supplementary Fig. 2b). Inhibition of DHODH activity and proliferation of immune cells (T and B lymphocytes and macrophages), as well as decrease of cytokines (IL-17, IL-6 and RANKL) were comparable between PBE− and PBE+ rats (Fig. 2b and Supplementary Fig. 2c). Inflammatory synovial hyperplasia was inhibited by Leflunomide in both PBE− and PBE+ rats (Supplementary Fig. 2d). The PBE+ rats demonstrated higher serum baseline CRP (CRP1, 91.24 ± 10.12 mg L−1) and TRAP5b, whereas the PBE− rats showed relatively lower CRP (CRPL, 52.45 ± 7.54 mg L−1) and TRAP5b (Fig. 2c). Leflunomide decreased serum CRP and TRAP5b levels and inhibited CRPH CIA rats but not in CRPH CIA rats. Hypoxia-inducible factor 1α (HIF1α) has been reported to compete with ARNT for ARNT association. We found that CRP upregulated HIF1α expression, which competed with ARNT for ARNT association and interfered Leflunomide-AHR-CRPH signaling in CRPH CIA rats. Knockdown of HIF1α re-activated Leflunomide-AHR-CRPH signaling in hepatocytes overexpressing CRP in vitro. Hepatocyte-specific deletion of HIF1α improved the Leflunomide-AHR-CRPH signaling to inhibit bone erosion in CRPH CIA mice. Acriflavine (ACF), a FDA-approved drug, has been reported as a selective inhibitor targeting HIF1α. We showed that ACF decreased binding of ARNT with HIF1α and facilitated Leflunomide activating AHR to inhibit CRP production and attenuate bone erosion in CRPH CIA rats with no obvious toxicity.

In summary, this study reveals that CRP-HIF1α signaling axis is responsible for the limited efficacy of Leflunomide in CRPH RA. On the basis of this finding, we develop a precision medicine-based therapeutic strategy for CRPH RA, i.e., the combination of Leflunomide and ACF.
bone resorption and osteoclastic activity in CRP\textsuperscript{L} rats but not in CRP\textsuperscript{H} rats (Fig. 2d, e, Supplementary Fig. 2d). We investigated the mechanism underlying the distinctive CRP levels between the two groups of RA individuals. IL-6 axis was the chief inducer of CRP expression in hepatocytes\textsuperscript{22}. We found that serum IL-6 were comparable between CRP\textsuperscript{L} and CRP\textsuperscript{H} rats (Supplementary Fig. 2c), while IL-6 receptor (IL-6R) in liver was differentially expressed between the two groups (Supplementary Fig. 2e).

**Attenuation of bone erosion by CRP inhibition in CIA rats.** The CRP\textsuperscript{H} CIA rats were intra-articularly injected with PBS (vehicle), IgG controls or anti-CRP antibodies (Supplementary Fig. 3a). The anti-CRP antibodies attenuated bone erosion and bone resorption and prevented bone loss in CRP\textsuperscript{H} rats when compared to IgG or PBS (Supplementary Fig. 3b–d). We also used an RNA interference-based strategy to inhibit hepatic CRP expression in CRP\textsuperscript{H} CIA rats. Lipid nanoparticles (LNPs) has been proven as liver-targeted delivery systems for siRNAs in vivo\textsuperscript{23,24}. The CRP\textsuperscript{H} CIA rats were intravenously administered with PBS (vehicle), LNPs, LNPs encapsulating negative control siRNA (LNPs-NC siRNA) or LNPs encapsulating CRP siRNA (LNPs-CRP siRNA) (Supplementary Fig. 3e). About 90% of the administered dose of LNPs-siRNA was found in liver (Supplementary Table 3). LNPs-CRP siRNA lowered CRP expression in hepatocytes, attenuated bone erosion and bone resorption and prevented bone loss in CRP\textsuperscript{H} rats when compared to PBS, LNPs or LNPs-NC siRNA (Supplementary Fig. 3f–i). We determined whether CRP knockdown by LNPs-siRNA could abrogate the differential responsiveness to Leflunomide between CRP\textsuperscript{H} and CRP\textsuperscript{L} CIA rats. Our results showed that Leflunomide in combination with LNPs-siRNA effectively attenuated bone resorption and prevented bone loss in CRP\textsuperscript{H} CIA rats.
Fig. 2 Differential responsiveness to Leflunomide among CIA rats. a The representative three-dimensional microCT images and clinical arthritic scores of the hind paws from PBE+ (n = 73) and PBE− (n = 77) CIA rats before (baseline, BL) and after Leflunomide treatment. Non-immunized (NI) rats (n = 20) were used as controls of the CIA rats. Scale bar, 5.0 mm. *P < 0.05 as determined by repeated-measures ANOVA with a post hoc test. b Relative levels of DHODH activity and proliferation of T and B lymphocytes in synovial fluid normalized to the corresponding baseline in PBE+ (n = 73) and PBE− (n = 77) CIA rats. *P < 0.05 as determined by one-way ANOVA with a post hoc test, NS: no significance. c The serum baseline CRP and TRAP5b levels in PBE− (n = 77) and PBE+ (n = 73) rats. CRP−: relatively lower CRP level (CRPlower, 52.45 ± 7.54 mg L−1). CRP+: higher CRP level (CRPhigher, 91.24 ± 10.12 mg L−1). *P < 0.05 as determined by two-sided t-test. d Relative changes of serum CRP (left) and TRAP5b (right) from the corresponding baseline in CRPH (n = 73) and CRPL (n = 77) rats. *P < 0.05 for day 14 or day 28 versus BL in CRP+ and CRP−. #P < 0.05 for CRP+ versus CRP− at day 14 and day 28, as determined by repeated-measures ANOVA with a post hoc test. e Bone resorption parameters including osteoclast surface per bone surface (Oc.S/BS) and osteoclast number per bone surface (Oc.N/BS) in CRPH (n = 73) and CRPL (n = 77) rats after normalization to the corresponding baseline. *P < 0.05 as determined by one-way ANOVA with a post-hoc test. Source data are provided as a Source Data file.

erosion in CRP+ CIA rats. The therapeutic efficacy of the combination in CRP+ CIA rats was equivalent with that of Leflunomide in CRP− CIA rats (Supplementary Fig. 4a–d). We also examined the effects of anti-CRP antibodies in CRP− CIA rats. The anti-CRP antibodies partially mimic the therapeutic efficacy of Leflunomide in attenuating bone erosion (Supplementary Fig. 4e–h).

Direct interaction between Leflunomide and AHR. AHR genomic signaling plays an essential role in controlling hepatic CRP production13. Upon stimulation with agonists, AHR binds to ARNT to inhibit CRP expression13,14,25. Accumulating evidence suggests that Leflunomide, rather than its metabolite A77 1726, could be an AHR agonist26. To confirm the direct interaction between Leflunomide and AHR, we incubated Flag-tagged AHR (Flag-AHR) with DMSO (vehicle), Leflunomide and A77 1726, respectively. After pull-down assay, we detected that AHR interacted with Leflunomide rather than A77 1726 (Fig. 3a). To identify the key residues involved in Leflunomide-AHR interaction, we conducted homology modeling of AHR PAS-A and PAS-B domains, which are required for dimerization with ARNT and interaction with agonists14. The optimal structure was chosen for molecular docking with Leflunomide (Supplementary Fig. 5a). Six binding modes were predicted (Supplementary Fig. 5b). Key residues in the best two binding modes including H291, K303, and V381 were chosen for mutation (Supplementary Table 4). Mutation of either H291 or K303 decreased the binding between Leflunomide and AHR, whereas mutation of V381 could not affect Leflunomide-AHR interaction. Mutation of both H291 and K303 resulted in a more significant decrease in Leflunomide-AHR interaction (Fig. 3b). We further conducted drug affinity...
responsive target stability (DARTS) assay to confirm the binding between AHR and Leflunomide. DARTS assay relies on the protection against proteolysis of the target proteins conferred by the interaction with small molecular drugs27. Flag-AHR, Flag-AHR mutants and BSA were incubated with Leflunomide or DMSO (vehicle), followed by digestion with proteases (subtilisin), respectively (Supplementary Fig. 6a). Mutation of either H291 or K303 rather than V381 significantly decreased the stability of AHR conferred by the interaction with Leflunomide, suggesting that both H291 and K303 participated in the interaction between Leflunomide and AHR. Mutation of both H291 and K303 lead to synergetic reduction of AHR stability conferred by the interaction with Leflunomide (Supplementary Fig. 6b).

**Leflunomide-AHR-CRP signaling in hepatocytes.** Human normal hepatocyte cell line (THLE-2) was incubated with DMSO (vehicle), Leflunomide and A77 1726, respectively. Immuno precipitation analysis showed that Leflunomide, rather than A77 1726, induced binding of AHR with ARNT in a dose-dependent manner (Fig. 3c). Level of CRP expression and release were significantly decreased by Leflunomide but not by A77 1726 (Fig. 3d). After AHR knockdown, Leflunomide could not decrease CRP expression and release (Supplementary Fig. 6c, d). Leflunomide is mainly metabolized to A77 1726 by a cytochrome P450 1A2 enzyme (CYP1A2) 28. Furafylline, a specific inhibitor of CYP1A2, could suppress metabolism of Leflunomide28. We incubated human normal primary hepatocytes with Leflunomide in the presence of Furafylline or not. Leflunomide induced binding of AHR with ARNT and decreased CRP expression and release. Stabilization of Leflunomide via inhibition of its metabolism by Furafylline further enhanced AHR-ARNT interaction and CRP inhibition (Supplementary Fig. 6e, f). All the above results demonstrated that Leflunomide itself but not A77 1726 induced AHR genomic signaling to inhibit CRP expression in normal hepatocytes in vitro. In addition to the genomic signaling,
AHR also functions through nongenomic pathways, which is independent of AHR-ARNT interaction. Some ligands have been reported to trigger AHR nongenomic pathways to regulate activities of certain proteins, such as Src, JunD, and cyclin A. Leflunomide had no effects on expression of JunD and cyclin A and Src activation (phosphorylation of Src) in THLE-2 cells (Supplementary Fig. 6g), indicating that Leflunomide specifically induced AHR genomic signaling in normal hepatocytes. To examine whether Leflunomide induced AHR genomic signaling to inhibit CRP in hepatocytes overexpressing CRP, THLE-2 cells were pretreated with IL-6 to induce overexpression of CRP. Leflunomide showed weak ability in induction of AHR-ARNT interaction in THLE-2 cells overexpressing CRP. Knockdown of CRP recovered the ability of Leflunomide in induction of AHR-ARNT interaction (Fig. 3e). Leflunomide could not inhibit CRP expression and release in THLE-2 cells overexpressing CRP (Fig. 3f).

Inhibition of CRP and bone erosion by Leflunomide itself.
Both CRPL and CRPH CIA rats were orally administered with vehicle, Leflunomide and A77 1726, respectively (Supplementary Fig. 7a). Levels of serum CRP and TRAP5b were lower in CRPL rats treated with Leflunomide than those treated with A77 1726 (Supplementary Fig. 7b). Inhibition of bone resorption and bone loss by Leflunomide was more significant in CRPL rats when compared to A77 1726 (Supplementary Fig. 7c, d). In contrast, Leflunomide could not significantly decrease CRP, bone resorption and bone loss in CRPH rats when compared to A77 1726 (Supplementary Fig. 7e-g). These results, together with the above data, lead us to postulate that besides the well-known immunomodulatory action of Leflunomide via A77 1726, Leflunomide itself could activate AHR genomic signaling to inhibit CRP production and then attenuate bone erosion in CRPL RA, whereas the Leflunomide-AHR-CRP signaling was dysfunctional in CRPH RA (Fig. 3g).

HIF1α-induced dysfunction of Leflunomide-AHR-CRP signaling. HIF1α has been reported to compete with AHR for ARNT association. We examined the level of HIF1α in hepatocytes from non-immunized, CRPL and CRPH CIA rats. CRPL rats had slightly elevated HIF1α expression when compared to non-immunized rats, while HIF1α level in CRPH rats was significantly increased (Fig. 4a). There was a positive association between CRP and HIF1α expression in both CRPL and CRPH rats (Supplementary Fig. 8a). In addition, CRP inhibition by LNP-CRP siRNA led to a reduction of HIF1α expression in hepatocytes from CRPL rats (Supplementary Fig. 8b). We manipulated CRP by siRNA or overexpressing vectors in THLE-2 cells in vitro. Knockdown of CRP decreased HIF1α expression and binding of ARNT with HIF1α (Fig. 4b, c, Supplementary Fig. 8c). THLE-2 cells with CRP overexpression showed increase of HIF1α and binding of ARNT with HIF1α (Fig. 4d, e, Supplementary Fig. 8d). We examined AHR genomic signaling stimulated by Leflunomide in THLE-2 cells transfected with CRP siRNA or overexpressing vectors. Knockdown of CRP enhanced AHR-ARNT interaction, whereas CRP overexpression reduced the interaction between ARNT and AHR in THLE-2 cells upon Leflunomide stimulation (Fig. 4f, g). Binding of AHR with ARNT and inhibition of CRP expression induced by Leflunomide was remarkable in hepatocytes from CRPL CIA rats but insignificant in hepatocytes from CRPH CIA rats (Supplementary Fig. 8e, f). We concluded that high expression of CRP could trigger upregulation of HIF1α, which competed with AHR for ARNT association, leading to the dysfunction of Leflunomide-AHR-CRP signaling in CRPH RA (Fig. 4h). We determined whether knockdown of HIF1α could improve Leflunomide-AHR-CRP signaling in THLE-2 cells overexpressing CRP in vitro. Knockdown of HIF1α decreased the binding of ARNT with HIF1α (Fig. 4i). HIF1α siRNA alone had no effects on AHR-ARNT interaction and CRP level (Fig. 4j, k). However, HIF1α siRNA in combination with Leflunomide effectively enhanced AHR genomic signaling and CRP inhibition (Fig. 4j, k), suggesting that inhibition of HIF1α could facilitate Leflunomide activating AHR to inhibit CRP expression in hepatocytes in CRPH RA.

Limited efficacy of Leflunomide in CRPH CIA mice. DBA/1 mice with CIA were administered with Leflunomide for 28 days (Supplementary Fig. 9a). The mice were also classified into PBE+ and PBE− subgroups based on microCT analysis. The deteriorated bone microarchitecture was observed in PBE+ CIA mice rather than in PBE− CIA mice after Leflunomide treatment (Supplementary Fig. 9b). Inhibition of DHODH activity and proliferation of immune cells (T and B lymphocytes and macrophages), as well as decrease of cytokines (IL-17, IL-6, and RANKL) were comparable between PBE− and PBE+ mice (Supplementary Fig. 9c). The PBE+ mice demonstrated higher serum baseline CRP (CRPH, 34.67 ± 5.41 mg L−1) and TRAP5b, whereas the PBE− mice showed relatively lower CRP (CRPL, 17.70 ± 3.25 mg L−1) and TRAP5b (Supplementary Fig. 9d). Leflunomide decreased levels of serum CRP and TRAP5b and inhibited bone resorption in CRPL mice but not in CRPH mice (Supplementary Fig. 9e, f).

Re-activation of Leflunomide by hepatic HIF1α deletion. HIF1αloxP/loxP mice were bred to Alb-cre mice to generate hepatocyte-specific HIF1α knockout (HIF1α−HKO) mice (Supplementary Fig. 10a, b). Serum CRP level was comparable between HIF1α−HKO and control (HIF1αloxP/loxP) mice (Supplementary Fig. 10c), suggesting that HIF1α could not affect CRP expression in hepatocytes in vivo. CIA was established in both HIF1α−HKO and control mice. They were classified into subgroups based on the above established reference range of CRP for CRPL (17.70 ± 3.25 mg L−1) and CRPH CIA mice (34.67 ± 5.41 mg L−1). After Leflunomide treatment, we observed the attenuated bone erosion in CRPL but not in CRPH control mice. However, both CRPL and CRPH HIF1α−HKO mice demonstrated satisfactory response to Leflunomide in attenuating bone erosion (Supplementary Fig. 10d, e). Inhibition of inflammatory synovial hyperplasia by Leflunomide in CRPL and CRPH groups was comparable for both control and HIF1α−HKO CIA rats (Supplementary Fig. 10f). Leflunomide decreased CRP and TRAP5b and inhibited osteoclastic activity and bone resorption in CRPL but not in CRPH control mice, while decrease of CRP and TRAP5b and inhibition of osteoclastic activity and bone resorption by Leflunomide were significant in both CRPL and CRPH HIF1α−HKO CIA rats (Supplementary Fig. 10g-i). All the above data suggested that HIF1α in hepatocyte was a molecular target for re-activating Leflunomide inhibiting CRP and bone erosion in CRPH RA.

Re-activation of Leflunomide-AHR-CRP signaling by ACF. THLE-2 cells overexpressing CRP were treated with DMSO (vehicle) or a HIF1α inhibitor ACF. ACF reduced binding of ARNT with HIF1α and downregulated the most classical target gene of HIF1α-ARNT signaling, i.e., vascular endothelial growth factor (VEGF) (Fig. 5a). THLE-2 cells overexpressing CRP were incubated with DMSO (vehicle), Leflunomide, ACF and combination of Leflunomide and ACF, respectively. The combination of Leflunomide and ACF enhanced binding of ARNT with AHR and reduced CRP expression and release when compared to Leflunomide or ACF.
alone (Fig. 5b, c). We collected the conditioned media from the above treatment groups and incubated the monocytes with the conditioned media in the presence of macrophage colony stimulating factor (M-CSF) (Fig. 5d). Osteoclast differentiation were inhibited in monocytes treated with conditioned medium from the combination of Leflunomide and ACF, but not in those cells treated with conditioned medium from Leflunomide or ACF alone group (Fig. 5e-g). These results demonstrated that ACF facilitated Leflunomide activating AHR to suppress CRP expression and decrease osteoclast differentiation in vitro.

Decrease of bone erosion by Leflunomide combined with ACF.
To investigate whether ACF facilitated Leflunomide decreasing CRP production and attenuating bone erosion in CRP^H CIA rats, CRP^H rats were administrated with vehicle, Leflunomide, ACF and the combination of Leflunomide and ACF, respectively (Fig. 6a). CRP^H rats administered with the combination of Leflunomide and ACF showed reduced serum CRP, attenuated bone erosion and bone resorption and prevented bone loss, whereas no obvious decrease of CRP and bone erosion were observed in CRP^H rats administered with Leflunomide or ACF alone (Fig. 6b–e and Supplementary Fig. 11). Leflunomide or the combination of Leflunomide and ACF attenuated inflammatory synovial hyperplasia in CRP^H CIA rats (Supplementary Fig. 11). There was no significant change in liver function parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), a kidney function parameter blood urea nitrogen (BUN) and hematology parameters including total protein (TP), hemoglobin, white blood cell (WBC), red blood cell (RBC), and platelet (PLT) in CRP^H CIA rats treated with the combination of Leflunomide and ACF when compared to Leflunomide or ACF alone (Supplementary Table 5).

Discussion
In our study, we found that Leflunomide exerts significant immunosuppression but limited efficacy in attenuating bone erosion in
RA individuals (PBE+) distinguished by higher serum CRP (CRP⁺), whereas the others (PBE−) with satisfactory responsiveness to Leflunomide show relatively lower serum CRP (CRP⁻).

The exact role of CRP in RA has been an unsettled dispute for a long time. No human study to date has directly investigated the contribution of CRP in RA, and animal studies performed so far have shown mixed results. CRP has been reported to induce osteoclastogenesis, indicating that CRP makes a detrimental contribution in RA. However, transgenic expression of human CRP in RA, and animal studies performed so far reveal the different roles of CRP in distinct stages of RA development (early induction stage versus late active stage). Together, they suggest that CRP may play a detrimental role during active RA, which is consistent with the assumption in one of the above studies.

Regarding the studies demonstrating the protective role of CRP in RA, CRP expression is manipulated prior to arthritic induction in genetic models (CRP transgenic and knockout mice). For our work and other studies demonstrating the detrimental role of CRP in RA, however, CRP level is influenced after arthritic induction in CIA models. The above studies actually show that CRP may have double-faced functions in RA.

Previously, Kim et al. report that CRP promotes osteoclastic activities via FcγRs signaling with the absence of RANKL, while Cho et al. show that CRP inhibits RANKL-induced osteoclastic activities in vitro. Recently, Jia et al. suggest that CRP regulates osteoclastic differentiation in conformation-
and RANKL-dependent manners. Circulating native CRP is composed of five identical subunits but dissociates into the monomeric conformation upon entering local lesions. Jia et al. show that monomeric CRP could induce osteoclastic differentiation with the absence of RANKL in vitro, which is consistent with Kim’s report. However, Jia et al. also demonstrate that monomeric CRP inhibits RANKL-induced osteoclastic differentiation by neutralizing RANKL in vitro, which is consistent and accounts for Cho’s findings. Jia et al. further explain that native CRP is inefficient for osteoclastic activity in vitro but the prolonged incubation time (3–21 days) in cell culture would favor the conversion of native CRP to monomeric CRP, suggesting that in both Kim and Cho’s studies, CRP regulates osteoclastic activity actually via the monomeric conformation, as they incubate cells with CRP for 3–21 days and 3–5 days, respectively. Based on these studies, we think whether CRP promotes or inhibits osteoclastic activities in vivo may be dependent on ratio of monomeric CRP to RANKL. We find that monol concentration of the monomeric CRP is over 10,000-fold of RANKL in both CRP\textsuperscript{L} and CRP\textsuperscript{H} RA patients, indicating that the monomeric CRP is enough to neutralizing RANKL and the redundant free monomeric CRP would dominate osteoclastic activities in RA.

Previously, pertinent publications frequently reiterate that mouse CRP is a trace protein and serum levels of CRP in mice are markedly lower than in humans and rabbits, \(<3\text{mg}\text{mL}^{-1}\) even after inflammatory stimuli. However, recent studies argue that such statements are not convincing as they either refer to review articles devoid of experimental data or an early publication, which fails to detect any mouse CRP using sheep anti-human CRP antibodies cross-reacting with mouse CRP before acute phase induction. To date, reliable reagents specific for mouse have become available and studies suggest that serum CRP levels may be vastly underestimated. High serum CRP levels ranging from 5 mg mL\(^{-1}\) to 50 mg mL\(^{-1}\) have been determined in different mouse models, which are consistent with our results.

In our work, Sprague-Dawley (SD) rats and DBA/1 mice are used for establishment of CIA, as they are common animal models for exploring the pathogenic mechanism of RA and therapeutic action of anti-arthritic agents. SD rat is an outbred rat is an inbred strain and mice are the basis for classification of CIA models into CRP\textsuperscript{L} and CRP\textsuperscript{H} subgroups. For many decades, inbred rats and mice are preferred over outbred strains because it is assumed that...
they display lower levels of variations due to their greater homogeneity\textsuperscript{50}. However, very few studies with adequate sample sizes have in fact compared phenotypic variations between inbred strains and outbred strains\textsuperscript{31}. Surprisingly, recent reports suggest that inbred and outbred animals show comparable phenotypic variations\textsuperscript{28-32}. On one hand, they conclude that outbred animals could be widely adopted as research subjects like inbred animals. On the other hand, it could not be ignored that the coefficients of variation vary in large ranges for both of them\textsuperscript{31}. We review other studies, which also show large variations of serum CRP \textsuperscript{42} and variations of proteins in established animal models seem to differ from the common sense and the underlying reasons are still unknown, the variations could be used for explaining non-response of therapeutics in human disease\textsuperscript{31}.

**Methods**

**Characteristics of RA patients treated with Leflunomide.** 250 RA patients (150 women and 100 men) were treated with Leflunomide. The characteristics of the patients before treatment were shown in Supplementary Table 1. The patients with other diseases or medication, including calcium supplements and postmenopausal hormone replacement therapy, that might affect bone remodeling, were excluded from the study. Female patients who were postmenopausal for fewer than 5 years were also excluded from the study. Informed consent was obtained from all patients. The study was approved by the Ethics Committee in Institute of Basic Research in Clinical medicine, China Academy of Chinese Medical Sciences (CACMS). All the included RA patients were orally administrated with Leflunomide for 12 months. The serum from all the RA patients was collected before and after treatment. The anteroposterior plain radiographs of hands were taken before and after treatment.

**Radiological evaluation by modified Sharp’s method.** The anteroposterior plain radiographs of the hands were read and evaluated by two different qualified doctors. The reader of the radiographs was blind to the order of the radiographs and the clinical details of the RA patients. The erosion score per joint of the hands was performed according to the modified Sharp’s score system. Briefly, the erosions for 32 joints of both hands were evaluated based on a 6-points scale (0-9). Erosions were scored 0 if there was no erosion. Erosions were scored 1 if they were discrete but clearly present and 2 or 3 if they were larger, depending on the surface area of the joint involved. A score of 3 was given if the erosion was large and extends over the imaginary middle of the bone. A score of 5 was given if a complete collapse of the joint was present or if the full surface of the joint is affected\textsuperscript{51}.

**Cell culture.** Human normal hepatocyte cell line THLE-2 (ATCC, CRL-2706) was cultured at 37 °C in a humidified environment containing 5% CO\textsubscript{2} and 20% O\textsubscript{2} in a humidified environment containing 5% CO\textsubscript{2} and 20% O\textsubscript{2} in an atmosphere containing 5% CO\textsubscript{2} and 20% O\textsubscript{2} in an atmosphere containing 5% CO\textsubscript{2} and 20% O\textsubscript{2}. Hundred microliter aliquots were mixed with 150 μL of 0.1% toluidine blue solution. Bone slice images were transferred onto PVDF membranes (Bio-Rad). After blocking, the membranes were probed with primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Immunodetection was performed using enhanced chemiluminescence (Thermo Fisher Scientific). β-actin was used as a loading control for internal correction. The primary antibodies included an anti-AHR antibody (ab2769, 1: 500), an anti-ARNT antibody (ab5624, 1: 500), an anti-HIF1α (ab1, 1: 200) antibody, an anti-CRP antibody (ab50861, 1: 2000), an anti-VEGFA antibody (ab46154, 1: 1000), an anti-JunD antibody (ab28357, 1: 500), an anti-cyclin A antibody (ab181591, 1: 2000), an anti-Src antibody (ab34705, 1: 500), an anti-α-Src (phospho Y418) antibody (ab84161, 1: 1000) and an anti-β-actin (ab8226, 1: 10000) antibody from Abcam. Antibodies were validated for the specific species and application in pilot studies. All relevant uncropped blots are available in the Data S1 file.

**Isolation of lymphocytes and macrophages.** The synovial fluid was obtained from RA patients, CIA rats and mice. T lymphocytes were isolated by EasySep™ T Cell Isolation Kits (STEMCELL Technologies Inc.). B lymphocytes were isolated by EasySep™ B Cell Isolation Kits (STEMCELL Technologies Inc.). The purity of T and B lymphocytes as determined by flow cytometry was approximately 90–95%. Macrophages were isolated by flow cytometry using an anti-CD14 antibody (17000-1-AP, 1: 100, Proteintech Group, Inc.).

**DHODH activity assay.** The assay comprised enzymatic reaction of DHODH with dihydorooric acid (DHOO) substrate, followed by fluorescence detection specific for orotic acid using the 4-trifluoromethylbenzamidoxime (4-TFMBAO) fluorogenic reagent\textsuperscript{45}. Briefly, 3 × 10\textsuperscript{5} lymphocytes were lysed by sonication for 10 min at 4 °C. Lysates were incubated with 1.0 ml aqueous solution containing 500 μM DHO, 200 mM K\textsubscript{2}CO\textsubscript{3}-HCl (pH 8.0), 0.2% Triton X-100 and 100 μM coenzyme Q10 at 37 °C for 4 h. After incubation, the preexisting level of orotic acid at non-incubation time was subtracted from total amount of orotic acid observed at the end of the hour incubation. Orotic acid, coenzyme Q10, 4-TFMBAO and DHO were purchased from Sigma-Aldrich.

**Isolation and osteoclastogenesis of human monocytes.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood of healthy volunteers (density gradient separation by density Ficoll-400; Sigma-Aldrich). The cells were washed three times with sterile PBS and resuspended in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin (complete medium). The PBMCs were incubated at 37 °C for 1 h in complete medium and allowed to adhere for 45 min. The nonadherent cells were removed and the adherent cells were washed with sterile PBS and harvested with a rubber policeman. The purity of the monocytes isolated by Ficoll-Hypaque as determined by flow cytometry was approximately 85–90%. The cells were cultured for three weeks in conditioned mediums from THLE-2 cells in the presence of 25 ng ml\textsuperscript{-1} recombinant human M-CSF (R&D Systems). The medium was changed every other day. On day 21, TRAP-positive cells were identified using a leucylcysteine phosphate kit (Sigma-Aldrich)\textsuperscript{56}.

**Isolation of hepatocytes.** Rat and mouse primary hepatocytes were isolated using a two-step perfusion process\textsuperscript{57}. Briefly, hepatocytes are dissociated from anesthetized adult rats by a non-recirculating collagenase perfusion through the portal vein. The isolated cells are then filtered through a 100 μm pore size mesh nylon filter. Cell number within the cell suspension was counted by using a hemocytometer. Cell viability was determined by trypan blue staining. Flow cytometry was used for purification and identification of the isolated hepatocytes.

**Pit formation assay.** Human monocytes were seeded on OsteoAssay bone plates (Lonza) at a density of 1 × 10\textsuperscript{6} cells per well. The cells were cultured for three weeks in conditioned mediums from THLE-2 cells in the presence of 25 ng ml\textsuperscript{-1} recombinant human M-CSF (R&D Systems). The medium was changed every other day. On day 21, bone slices were ultra-sonicated in 1 M NH\textsubscript{4}OH to remove adherent cells and stained with 0.1% toluidine blue solution. Bone slice images were captured using electron microscopy. Three fields were randomly selected for each...
bone slice for further analysis. Pit areas were quantified using Image Pro Plus 6.2 software (Media Cybernetics Inc.)

Molecular docking. Position-Specific Iterated BLAST (PSI-BLAST) against PDB database was performed to identify homology templates of AHR PAS-α and PAS-B domains. Homology modeling were performed by MODELER software to build 3D structure of AHR PAS-α and PAS-B domains. Structure with lowest energy was chosen as the starting point for further structure refinements. In order to find optimized conformations, loop refinement was applied to the predicted structure of AHR PAS-α and PAS-B domain. The final model was assessed by the quality of modeled structure by inspecting backbone dihedral angles ϕ against ψ of amino acid residues in protein structure. Molecular docking was conducted between AHR PAS-α and PAS-B domains and Leflunomide using AutoDock Vina. Once the macromolecule structure of AHR is loaded, charges and hydrogen atoms were added, and non-polar hydrogen atoms were merged. Leflunomide molecule was drawn using ChemDraw 10.0 and structure were optimized using quantum chemistry method AM1. Both protein and ligand structures were converted to PDBQT format using AutoDock tools.

ELISA assay. The human and rat CRP were measured by ELISA kits from Thermo Fisher Scientific. The mouse CRP was measured by a Quantikine ELISA kit (R&D Systems). The human rheumatoid factors including IGA, IGM and IGG were determined by a PeliKine human ELISA kit from Cell Sciences and an ELISA kit from Fisher Scientific. The human, rat and mouse IL-6 was determined by ELISA kits from Immunodiagnostic Systems, respectively. The human, rat and mouse CRP were measured by ELISA kits from Immunodiagnostic Systems, respectively. The human and rat CRP were determined by ELISA kits from Boster Biological Technology, respectively. The human and rat IL-6 were determined by ELISA kits from Cusabio Biotech. The human IL-6 and RANKL were determined by a PelikineTM human ELISA kit from Cell Sciences and an ELISA kit from Boster Biological Technology, respectively. The human, rat and mouse IL-6 was determined by ELISA kits from Abcam. The human TRAPs was determined by an ELISA kit from TECOmedical A. The mouse and rat TRAPs were determined by ELISA kits from Immunodiagnostic Systems, respectively. The human and rat IL-6 was determined by ELISA kits from Abcam. The mouse and rat RANKL were determined by ELISA kits from Abcam and Biobyt. The monomeric CRP was determined by an ELISA assay.

Serum biochemistry and hematology assays. The rat liver function parameters (ALT and AST) and a kidney function parameter (BUN) were analyzed by a Vitros 250 Analyzer (Ortho Clinical Diagnostics, Johnson & Johnson Co, Rochester, NY). The hematological parameters including TP, hemoglobin, WBC, RBC and PLT were analyzed by an ABX Pentra 60 C + Analyzer (Horiba ABX, Montpellier, France).

Preparation of LNPs-siRNA. LNPs delivery systems comprised lipid-like 98N12-5 (1), cholesterol, PEG-lipid and siRNA12,24. Briefly, sodium acetate buffer (pH 5.2) and 35% ethanol was added to the nanoparticles at the total lipid:siRNA molar ratio of 2:1. The solution containing 35% ethanol, resulting in spontaneous formation of empty lipidoid nanoparticles. The resultant nanoparticles with Cy5 and/or FITC-labeled siRNA were used in the in vitro experiments. The Cy5-labeled siRNA or NC siRNA in 50 mM sodium acetate buffer (pH 5.2) and 35% ethanol was added to the nanoparticles at the total lipid:siRNA molar ratio of 7:1 (wt/vt) and incubated at 37 °C for 30 min. Ethanol removal and buffer exchange of siRNA-containing nanoparticles was achieved by dialysis against PBS, including 0.15% sodium acetate. Finally, the formulation was filtered through a 0.2 µm sterile filter. For determining tissue distribution of the siRNA, siRNA was labelled with Cy5 and fluorescence intensity in different organ samples was measured by a microplate reader system (BioScan, Washington, DC).

CIA rat model. Male SD rats (170–190 g of weight) were from the Chinese University of Hong Kong and kept with free access to food and water under standard temperature conditions (22 °C) and a 12 h light/dark cycle. CIA rat model was induced64. Briefly, bovine type II collagen (C II) (Chondrex) was emulsified in an equal volume of incomplete Freund’s adjuvant (IFA, Chondrex). The rats were immunized subcutaneously at the base of the tail with 200 μl emulsion (200 μg of bovine C II). Booster immunization was administered on day 7 with 100 μl injection of same emulsion as the first time. Arthritis severity was evaluated by clinical arthritic scores which were performed by two independent blinded observers. Scoring was performed with a 0–4 scale, where 0 = no swelling or erythema; 1 = slight swelling and/or erythema; 2 = low-to-moderate edema; 3 = pronounced edema with limited joint usage; and 4 = excess edema with joint rigidity. Each hind limb was graded and thus the maximum possible score was 8 for each rat. A rat with a score of one or above was regarded as arthritic. All the experimental procedures were approved by the Committee of Animals Ethics and Experimental Safety of the Hong Kong Baptist University. We complied with all relevant ethical regulations for animal testing and research.

Drugs administration. Leflunomide and ACF were dissolved in PBS and intraperitoneally administrated to the CIA rats or mice, respectively. ACF was dissolved in 10% neutral formalin, decalcified in an equal volume of Complete Freund’s adjuvant (CFA). The mice were immunized with subcutaneous injection of the tail with 100 μl emulsion containing 10 μg/ml of bovine C II) containing a final concentration of 2 mg ml−1 of Mycobacterium tuberculosis. Arthritis severity was evaluated by clinical arthritic scores which were performed by two independent, blinded observers. Scoring was performed with a 0–4 scale, where 0 = normal; 1 = redness and/or swelling in one joint; 2 = redness and/or swelling in more than one joint; 3 = redness and/or swelling in the entire paw; and 4 = deformity and/or ankylosis. Each hind limb was graded and thus the maximum possible score was 8 for each mouse. A mouse with a score of one or above was regarded as arthritic. All the experimental procedures were approved by the Committee of Animals Ethics and Experimental Safety of the Hong Kong Baptist University. We complied with all relevant ethical regulations for animal testing and research.

Histological analysis. Mouse and rat knee joints were removed postmortem, stored in 10% neutral formalin, decalcified in 20% ethylenediaminetetraacetic acid (EDTA) for 4 weeks, then dehydrated and embedded in paraffin. The sections were stained with hematoxylin & eosin (HE) and tartrate-resistant acid phosphatase (TRAP) before histopathological analysis.

MicroCT analysis. For in vivo microCT analysis, the rats or mice were anaesthetized and placed prone on a scan stage before scanning. A vivaCT 40 (Scanco Medical) was employed to scan a region of 20 mm in length covering the right paw and tarsal bones at a voltage of 70 keV with a current of 114 μA. For ex vivo microCT measurement, the bilateral hind paws were dissected for microCT measurement. After automatic reconstruction, 2D slices with 21 μm isotropic resolution were generated and used to select the region of interest (ROI) for 3D reconstruction. The whole bone tissue of calcaneus was subjected to quantitative evaluation with a threshold (sigma = 1.2, support = 2 and threshold = 200). Bone volume fraction (BV/TV) and bone mineral density (BMD) were calculated using built-in software.

Bone histomorphometric analysis. The bilateral hind paws were fixed in 70% ethanol. The whole skeletal tissues from bilateral hind paws were dehydrated in graded concentrations of ethanol and embedded without decalcification in modified methyl methacrylate. Frontal sections of the whole bone from the hind paws at a thickness of 10 μm was obtained using an EXAKT Cut/grinding System (EXAKT Technologies, Inc. Germany). Thereafter, the bone sections underwent bone histomorphometric analyses using professional image analysis software (BIOQUANT OSTE0 analysis software) under fluorescence microscope (Leica image analysis system, Q500MC). The bone resorption parameters, i.e. Oc/BS and Ocn/BS, were calculated and expressed according to standardized nomenclature for bone histomorphometry.

DARTS assay. Thirty microliter Flag-tagged AHR, AHR mutants and negative control BSA in 100 μL PBS was incubated with vehicle (DMSO) and Leflunomide at the indicated concentrations (5.0 and 10.0 μM) for 18 h at 4 °C, respectively. Flag-tagged proteins were collected by a FLAG® M purification kit (Sigma-Aldrich). The small molecules in pull-down assays were extracted by 90% methanol (Sigma-Aldrich) and analyzed by Agilent 6400 Ultra Trace quadrupole ion trap mass spectrometer. The mass spectrometric conditions were as follows: Ion spray voltage, 5500 V; source temperature, 500 °C; cone gas flow, 20 L/h; ion spray gas flow, 10 L/h; declustering potential, 150 V; entrance potential, 10 V; octapole potential, 10 V; collision intersection, 30%; collision energy, 10 eV. The precursor ions were selected for collision with argon gas at a collision energy of 10 eV.
Statistical analysis. The binary logistic regression was used to determine the
indication accuracies of blood biomarker indicators in PBE + RA patients.
The Pearson’s correlation was used to determine correlations between variables.
The Shapiro-Wilk W test was used to test for normality of all continuous variables.
When comparing measured outcomes in two independent groups, two-sided t-test
was used for analyzing continuous variables. When comparing repeated measured
outcomes in time series groups, repeated-measures analysis of variance (ANOVA)
was used for analyzing the data. For difference among multiple independent
groups, one-way ANOVA with a post-hoc test was performed. Samples were ran-
domly located into different groups. The investigators were blinded to group
allocation during data collection and analysis. A two-sided P-value of less than 0.05
was considered statistically significant. All statistical analyses were performed with
a SPSS software, version 22.0. Two types of replicates including biological replicates
and technical replicates was performed to yield accurate and reliable statistics.

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Author contributions

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Additional information

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