TNFα inhibitors reduce bone loss in rheumatoid arthritis independent of clinical response by reducing osteoclast precursors and IL-20

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Key messages:

- TNFα inhibitors reduce bone loss in RA independent of DAS28 improvement through curbing osteoclastogenesis.
- TNFα inhibitors reduce bone loss in RA through reducing RANK expression and IL-20 production.

ABSTRACT

Objectives: About half of rheumatoid arthritis (RA) patients treated with TNFα inhibitors either do not respond or lose their initial therapeutic response over time. The clinical response is measured by reduction in DAS28, which primarily reflects inflammation. However, other effects of TNFα inhibitors, such as impact on bone erosion, are not assessed by DAS28. We examined the effect of TNFα inhibitors on bone density, bone biomarkers and cytokine production in responder and non-responder patients and assessed mechanisms of action.

Methods: Bone mineral density (BMD) in the lumbar spine and femur neck of 117 RA patients was measured by DEXA scan. Bone turnover biomarkers CTX, OPG, osteocalcin, and RANKL were measured by ELISA. Levels of 16 cytokines in plasma and in tissue culture supernatants of *ex vivo* T cells were measured by multiplex assays and ELISA. The effect of treatment with TNFα inhibitors on blood mononuclear cell (MNC) differentiation to osteoclast precursors (OCP) was measured flow cytometry and microscopy.

Results: TNFα inhibitors improved lumbar spine BMD but had modest effects on blood bone biomarkers, irrespective of patients' clinical response. Blood OCP numbers and the ability of monocytes to differentiate to OCP *in vitro* declined after treatment. Treatment also reduced RANK expression and IL-20 production. BMD improvement correlated with reduced levels of IL-20 in responder patients.

Conclusion: This study reveals that TNFα inhibitors reduce lumbar spine bone loss in RA patients irrespective of changes in DAS28. The reduction in bone loss is associated with reduction in IL-20 levels in responder patients.

Introduction

Rheumatoid arthritis (RA) pathology primarily affects joints causing chronic inflammation, joint destruction and bone erosion [1]. The disease is mediated by T cells and sustained by chronic production of pro-inflammatory mediators and tissue degrading enzymes. The principal pro-inflammatory mediator in most RA patients is TNFα to which biological therapies have been developed and successfully applied [2].

In addition to inflammation, RA manifests periarticular and generalised bone loss with a high risk of fractures [3]. The available evidence indicates that, in addition to driving chronic inflammation, TNFα promotes bone loss in RA patients. Studies of osteoporosis in aged individuals provides support for the role of TNFα in generalised bone loss [3,4]. Mechanistically, TNFα can promote bone loss through upregulating the expression of receptor activator of nuclear factor kappa-B (RANK) and its ligand, RANK-L [5]. In animal models, TNFα promotes osteoporosis by inducing osteoclast differentiation through enhancing RANK/RANK-L expression and reducing osteoprotegerin (OPG) production [6,7]. TNFα also inhibits osteoblasts and promotes their apoptosis by increasing the expression of the Wnt antagonist DKK1, all of which impair new bone formation [8]. Bone loss in RA starts early and progresses rapidly. Indeed, decreases of 2.5% in vertebra and 5% in femoral neck BMD are seen in the first year and these double in the second year if the disease is not controlled [9].

The evidence indicates that TNFα inhibitors reduce inflammation and bone erosion in RA [10]. However, it remains unclear whether these agents reduce bone loss in parallel with the clinical response or not. Thus, about 50% of RA patients receiving TNFα inhibitors either do not respond to treatment with these biologicals or relapse after an initial response and it is unclear if there is improvement to bone loss despite a lack of clinical response as assessed by changes

to the disease activity score 28 (DAS28) [11]. DAS28, is a composite score of the number of swollen joints (out of the 28), the number of tender joints, erythrocyte sedimentation rate (ESR) or C reactive protein (CRP) measurements and patients' 'global assessment of health'. DAS28, however, does not reflect bone loss or accurately reflect synovitis. This is because DAS28 includes subjective components, driven by factors such as chronic pain. Furthermore, it remains unclear how bone erosion is included in patient reporting that determine DAS28. In addition, the mechanism(s) and pathways through which TNFα inhibitors reduce bone loss, especially generalised bone loss are unclear. Studies of bone loss suggest that TNFα inhibitors can reduce osteoclastogenesis [12]. Indeed, in mice engineered genetically to lack osteoclasts, TNFα does not elicit bone loss [13]. Reduced osteoclastogenesis following treatment with TNFα inhibitors is suggested to be due to reduced RANK-L expression [14]. Interestingly, inhibition of RANK-L does not affect chronic inflammation [15].

Previous studies, including our studies, have shown that non-responder RA patients have high numbers of Th17 cells and that residual disease in TNFα inhibitor-treated patients could be driven by IL-17 [16]. Th17 cells provide immunity against extracellular bacteria and fungi but can also promote chronic inflammation. However, their role in bone loss is less clear than that of TNFα. For example, IL-17 can indirectly promote bone loss through inducing pro-erosion cytokines, mediate anti-apoptotic signals in synoviocytes and enhance inflammatory cell maturation [17,18]. Th17 cells also induce RANK-L and pro-inflammatory cytokines in osteoblasts [18-20]. Importantly, IL-17 also has a potent synergistic effect with TNFα [16,20,21].

The main aims of this study were to analyse the effect of TNFα inhibitors on bone loss in responder and non-responder RA patients and gain insights into potential mechanisms through which biologic TNFα inhibitors reduce bone loss.

Methods

Patients

RA patients were recruited from Barts Health NHS Trust rheumatology clinics. The study was carried out as a prospective study, approved by the City and East London Ethical Committee (06/Q0605/8; NRES Committee) and carried out in compliance with the Declaration of Helsinki 2013. The study is not a clinical trial and was carried out over a period of 4 years. Recruited patients were prescribed TNFα inhibitors by their rheumatologists based on their clinical needs. The patients were prescribed biologic TNFα inhibitors according on NICE guidelines. None of the non-responder patients identified at 3 months converted to become a responders after the 3 month time point. The data were collected and retained at Barts Health NHS Trust and the William Harvey Research Institute until analysed. The clinical response of individual patients was blinded to the investigators who carried out the laboratory tests and only revealed when data collection was completed. In all, 117 RA patients were recruited from those undergoing BMD measurements. Patients' responses were determined based on EULAR criteria; patients were considered responders when their DAS28 (using CRP test results) decreased by ≥1.2-3.2 after 3 months [22]. Twenty seven patients (23%) were males and 90 (77%) were females. The cohort had a mean and standard deviation (SD) age of 63.4±14.7 years. At 3 months after treatment, 83 (71%) patients responded while 24 (29%) did not. The mean±SD of age of responders and non-responders were 62.7±15.1 and 64.9±14.1 years, respectively (Table 1). The mean±SD of DAS28 of responders and non-responders prior to treatment were 5.8±0.9 and 6.1±0.7, respectively.

Twenty five mL heparinised blood samples were drawn at 3 time points from selected patients before and at 1 and 3 months after treatment. In parallel, 25mL blood samples were collected from 14 age/sex matched healthy controls (HCs) and used for the laboratory experiments. B

cells, T cells and monocytes were enriched by negative selection as described [23] (Supplementary Data S1). Changes in bone were determined by measuring bone mineral density (BMD) at 6 months-1 year and then at 2 years after treatment at both sites of the lumbar spine (L2-4) and hips (femoral neck) using A Hologic Discovery QDR series Dual Energy X-ray Absorptiometry (DEXA) and compared from before to after treatment. The same device was used throughout the study for all of the patients.

Assessment of the immune system in treated patients

Enriched immune cells were studied by FACS for membrane protein expression, intracellular cytokines and cytokine production after stimulation *in vitro* as described [23] (Supplementary Data S1). For cytokine quantification in plasma and in culture supernatants, multiplex MSD kits (MSD Technologies) were used. The following cytokines/chemokines were quantified: TNFα, IL-17, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-23p40, GM-CSF, IFNγ and MCP-1 and IL-22. Plasma RANK-L, OPG and IL-20 levels were quantified using ELISA kits (R&D Technologies). Plasma osteocalcin and C-telopeptide cross-links of collagen type I (CTX-I) were determined using ELISA.

Treatment with TNFα inhibitors and the frequency of circulating precursors of osteoclast (pOCL)

Peripheral blood mononuclear cells (PBMCs) from 8 patients and 8 matched HCs were separated, studied by FACS or cultured *in vitro* and stained with tartrate-resistant acid phosphatase (TRAP; Sigma) to determine the frequency of pOCL [24]. In addition, PBMCs from the patients and HCs were tested for their ability to differentiate to pOCL *in vitro* after 14 days culture and staining for TRAP+ multinucleated cells. Furthermore, the ability of factors produced by cultured PBMCs from RA patients on the differentiation of PBMCs from each of 4 HCs to pOCL were also tested. Finally, the effect of blocking TNFα in the RA supernatants

on the differentiation of PBMCs from HCs was tested. TRAP+ multinucleated cells, each containing ≥3 nuclei were enumerated and presented as pOCL/10^6 PBMCs from RA patients and HCs.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism software. Mann-Whitney U test, Student's t-test, Wilcoxon matched-pairs signed rank test and Spearman's correlation coefficient (r) were used for the analysis of differences between or within groups and correlations as appropriate.

Results

Blockade of TNFα improves lumbar spine BMD

A total of 117 RA patients treated with TNFα inhibitors were assessed for their BMD, 27 (23%) males and 90 (77%) females. The patients received either etanercept, certolizumab, adalimumab, infliximab or golimumab in combination with methotrexate. Fourteen patients (8 responders and 6 non-responders) were on prednisolone. Twenty five patients were taking calcium and vitamin D medications. Eighty three patients (71%) responded to the treatment at 3 months while 24 (29%) did not. There were no significant differences in age (64.5±5.3 vs. 62.3±10.3 years), ethnicity or gender between responders and non-responders. The mean±SD of DAS28 (using CRP) of responders and non-responders pre-treatment were 5.8±0.9 and 6.1±0.7 (P>0.05). No obvious or consistent differences in cytokine profiles in patients treated with different TNFα inhibitors were observed. Based on reports that the level of some pro-inflammatory cytokines increase with reduced levels of estrogen (E2), as in ovariectomized arthritic mice and postmenopausal women, we compared the level of TNFα, IL-17, IL-1β, IL-6 and GM-CSF in female patients for the effect of menopause on the level of these cytokines produced by T cells [25, 26]. The patients were divided pre- and post- menopause according to age into those 50 years of age or younger (≤50) and those older than 50 years, respectively. The analyses revealed no statistically significant differences in the level of the tested cytokines between the two groups although there was a trend for higher levels of IL-6 and, to a lesser extent, IL-1β and IL-17 in the >50 year age group (Supplementary Figure S1).

After 1 year of treatment, data available on patients assessed at this time point showed that there was improvement in the mean BMD of lumbar spine in responder patients from 0.853±0.08 to 0.863±0.09 g/cm^2 but not in non-responder patients, 0.849±0.06 to 0.838±0.05. However, at 2 years after treatment, both responder and non-responder patients displayed

improvements in their mean lumbar spine BMD, from 0.935±0.15 to 0.962±0.16 g/cm^2 (P<0.0001) in responders and from 0.885±0.10 to 0.914±0.12 g/cm^2 (P=0.0773) in non-responders (Table 1). The improvements were irrespective of clinical responses at 3 months as measured by changes to DAS28. However, there was no, or minor improvements in BMD of the hips in either group. Therefore, all data described in relation to BMD in this report relate to the lumbar region.

More male patients showed improvement to their lumbar spine BMD than female patients; 90% vs. 58%. In non-responders, 67% of the males had BMD improvement compared with 50% of the females. There were also higher rates of BMD improvement in younger patients, 88% of ≤50 year compared with 61% >50 year patients.

Cytokine profiles and changes in bone parameters after treatment with TNFα inhibitors

Data on levels of cytokines in the treated patients were consistent with our previous studies. Thus, the current study showed that T cells from responder patients produced higher levels of TNFα and GM-CSF before treatment when stimulated *in vitro* compared with non-responder patients (Fig. 1). In contrast, higher levels of IL-17 were produced by T cells from non-responders when stimulated *in vitro* compared with responders [16,23]. In addition, T cells from responder patients produced higher levels of IL-1β and IL-6 *in vitro* compared with non-responders. High levels of TNFα and IL-1β, produced by T cells *in vitro* inversely correlated with BMD pre-treatment (R^2=0.48 and 0.09). However, high levels of IL-17 in non-responder patients did not correlate with BMD (not shown). Plasma IL-20 levels were significantly higher in responder patients (20.0±30.1 ng/mL) pre-treatment than in non-responders (1.8±2.6 ng/mL; P<0.05) (Fig. 2).

After treatment, there were no major changes in cytokine or bone biomarker levels in the first 3 months with few exceptions (Fig. 2). For example, TNFα produced by T cells *in vitro* decreased in responders while IL-1β and IL-17 increased after 1 month in responders (Fig. 2A-C). Plasma IL-20 levels in responders declined from 20.1±6.9 ng/mL to 15.8±25.0 ng/mL and 12.4±19.7 ng/mL after 1 and 3 months of treatment, respectively (Fig. 2D). This paralleled a decrease in RANK expression on monocytes. Plasma levels of IL-22 in responders and non-responders pre-treatment were lower than IL-20, at 5.2±3.4 ng/mL and 0.91±0.9 ng/mL, respectively (non-significant). In contrast to IL-20, IL-22 levels did not change significantly in responders after treatment, 4.3±2.4 ng/mL after 1 month and 4.7±3.8 ng/mL 3 months. There was a significant correlation between plasma TNFα and IL-20 levels in T cell culture supernatants from responder patients (P= 0.0002; Supplementary Figure S2, A). There was also a correlation between TNFα and IL-20 produced by T cells though this was not statistically significant (P= 0.06; Supplementary Figure S2, B). There were no notable changes in the level of most of the bone biomarkers after treatment with the exception of a small increase in RANK-L in non-responders after 1 month and a marginal increase in osteocalcin in responders at 3 months (Fig. 2E and H). There was a weak correlation between plasma CTX and TNFα in stimulated T cell culture supernatants (Fig. 3A). In contrast, there was an inverse correlation between osteocalcin and osteoprotegerin (OPG) and TNFα in T cell culture supernatants (Fig. 3B, C). There was also a correlation between IL-6 in T cell culture supernatants and CTX pre-treatment (Fig. 3D) and an inverse correlation with OPG although these correlations appear to be influenced by the outliner data from some of the patients (Fig. 3E). When improvements in BMD after 2 years were correlated with changes in cytokine and bone biomarker levels at 3 months after treatment, there was only a significant correlation between decreased levels of TNFα in responder patients and improved BMD (P= 0.0057). There was also a trend for

reduced IL-1β levels in correlation with improved BMD but this correlation was not statistically significant (P= 0.11).

Treatment with TNFα inhibitors reduce the frequency of pOCLs

The frequency of pOCLs in the blood of HCs and RA patients before and after treatment was determined by FACS [24]. In addition, the ability of monocytes in PBMCs from the HCs and patients to differentiate to pOCLs *in vitro* and the effect of infliximab on the differentiation before and after treatment *in vitro* was assessed.

The frequency of $CD11b^{+}CD14^{+}$ monocytes in PBMCs of RA patients increased from 11.3±5.7% before treatment to 15.5±6.0% after 1 month and to 16.9±8.1% after 3 months of treatment (Fig 4A). The mean fluorescence intensity (MFI) of RANK expression on monocytes decreased from 634.5±302.7 pre-treatment to 399.6±155.5 and 289.1±99.9 after 1 and 3 months of treatment, respectively (Fig. 4B). *In vitro* cultures of PBMCs showed a significantly higher tendency of monocytes from the patients before treatment to differentiate to $TRAP^{+}$ pOCLs compared with PBMCs from the HCs (P=0.0002) (Fig. 4C-E). Thus, 40.6±5.6 $TRAP^{+}$ pOCLs were detected in cultures of PBMCs from patients pre-treatment compared with 11.4±3.3 from the HCs' PBMCs. After treatment, the number of pOCLs in cultures of PBMCs from the patients declined from $40.6\pm5.6/10^{6}$ before treatment to 24.3±3.1 after 1 month and 25.2±3.6 after 3 months (P<0.05) (Fig. 4F).

To determine whether the treatment directly influenced the differentiation of PBMCs to $TRAP^{+}$ pOCLs, we established a culture system in which PBMCs from 6 HCs were individually cultured with pooled supernatants from the culture of PBMCs from 10 RA patients pre-treatment. The PBMCs from the HCs were cultured with and without infliximab for 14 days and the number of $TRAP^{+}$ pOCLs determined. As positive controls, PBMCs from the HCs

were cultured with recombinant RANK-L and M-CSF (Fig. 5A and B) [27]. Culture of HC PBMCs with RANK-L and M-CSF increased pOCLs from 20.3±7.7 cells/10^6 PBMCs to 222.5±64.1 cells/10^6 PBMCs (Fig. 5C). Culture of PBMCs from the HCs with the pooled RA supernatant increased the number of pOCLs from 20.3±7.7 cells/10^6 to 65.0±14.1 cells/10^6 PBMCs. The addition of infliximab (at 2.5μg/mL) reduced the number of $TRAP^+$ pOCLs from 66.7±10.4 to 5.0±4.1 cells/10^6 PBMCs (P<0.0001) (Fig. 5D). The addition of OPG (1μg/mL) to RA PBMC significantly reduced the number of pOCLs after 14 days of culture from 45.1±10.9 cells to 15.0±3.5; the combination of OPG and infliximab further reduced the number of pOCLs to 5.0±4.1 cells/10^6 PBMCs (P<0.05) (Fig. 5E).

Discussion

The discovery and application of biological TNFα inhibitors have revolutionised medical care for RA patients. In addition to ameliorating chronic inflammation, there is evidence that these agents, as are some other biological therapies, can reduce or even reverse bone loss [28-30]. However, the exact relationship between the clinical response and bone loss and also mechanisms by which these agents reduce bone loss is unclear. The clinical response of RA patients is measured by reduction in DAS28. However, DAS28 includes subjective components and does not accurately reflect changes in bone. The latter inference is important as approximately half of the treated patients either do not respond or relapse after an initial response to TNFα inhibitors as assessed by DAS28 which means that the effect of the treatment on bone loss could not be evaluated in non-responders. In addition, studies in a number of laboratories including ours have demonstrated that the disease in non-responder RA patients is likely to be driven IL-17 which has a complex relationship with TNFα and bone [16]. For example, both cytokines induce the production of each other [31]. There is also evidence that both promote synovial inflammation differently [20]. For bone loss, however, the current evidence suggests that bone loss by IL-17 is likely to be mediated through TNFα [32]. It is, however, established that combined inhibition of TNFα and IL-17 is more effective than TNFα alone in controlling bone resorption [33].

The current study reveals that TNFα plays a key role in generalised bone loss in RA patients independent of whether patients respond clinically or not to treatment with TNFα inhibitors. The pre-treatment data show an inverse relationship between high TNFα levels and low BMD in all patients. The data also show a correlation between plasma CTX and an inverse relationship of OPG with TNFα levels. In contrast, levels of IL-17 did not correlate with BMD. The relationship between high TNFα levels and low BMD has been reported before [34]. This

relationship was substantiated by the reported arrest in bone loss in patients treated with a TNFα inhibitor (infliximab) [35]. In contrast to TNFα, IL-17 has a more selective effect on cortical bone through its effect on osteoblast apoptosis, reducing the number of bone lining cells and Wnt10b expression [36]. Thus, the evidence indicates that IL-17 plays a complex role in bone metabolism including an ability to promote new bone formation [37]. IL-17 causes bone resorption through enhancing RANKL expression and inhibition of Wnt signalling [38]. However, there is also evidence that IL-17 can promote new bone formation in animal models and human primary cells [39]. IL-17 has also been reported to boost new bone formation via enhancing osteoblast differentiation from mesenchymal stem cells. Further studies are required to determine the complex role that IL-17 could be playing in bone loss in RA patients as compared, for example, to patients with ankylosing spondylitis. For example, it will be of interest to determine whether blocking TNFα in non-responder RA patients could augment the role of IL-17 in promoting osteoblast differentiation in non-responders treated with TNFα inhibitors. This may, at least partly, explain how treatment of non-responder patients with the TNFα inhibitors improve or stabilise BMD.

The study also explored potential mechanisms through which TNFα promotes while its blockade ameliorates bone loss. First, we measured levels of IL-20 and IL-22 in the blood and culture supernatants of *in vitro* activated T cells. These two cytokines are members of the IL-10 family of cytokines [40,41]. IL-20 is produced by monocytes and endothelial cells and promotes osteoclastogenesis by binding to one of two receptor complexes IL-20R1/R2 or IL-22R1/IL-20R2 [40,42]. IL-22, in contrast, is produced by Th17, Th22, γδ T cells and NK cells and functions by binding to its heterodimeric receptor that consists of IL-22R1 and IL-10R2 [41]. Both IL-20 and IL-22 expressed in the synovium of RA patients [41]**.** Furthermore, high levels of IL-22^{+} cells in the synovium are associated high DAS28 and can promote monocyte

differentiation to pOCLs in the absence of RANK-L [43]. Results from our study showed a statistical association between high levels of IL-20 and low BMD pre-treatment. In addition, the follow up studies showed that levels of IL-20 declined after treatment in responder RA patients suggesting that the cytokine could be involved bone loss due to TNFα and the promotion of pOCL differentiation. This is relevant to bone loss in RA since IL-20 downregulates OPG, the decoy receptor for RANK-L [42].

The study also examined the frequency of pOCLs in the blood of patients before and after treatment, the potential of PBMCs from patients to differentiate to pOCL and the effect on a TNFα inhibitor on this differentiation. The frequency of pOCLs in RA patients before treatment was significantly higher than HCs but decreased after treatment with the TNFα inhibitors. Expression of RANK on monocytes also decreased suggesting a reduced potential for RA PBMCs to respond to RANK-L binding. The effect of treatment on the differentiation of PBMCs to pOCLs was also studied *in vitro*. These experiments revealed a reduced ability of cultured RA PBMCs to differentiate to pOCL after treatment with TNFα inhibitors compared with before treatment. The experiments also revealed that TNFα produced by PBMCs from RA patients could be involved in the differentiation of PBMCs from healthy controls to pOCLs. Treatment of RA patients with TNFα inhibitors reduced the frequency of blood pOCL irrespective of patients' clinical response measured by DAS28. These observations were supported by results of TNFα inhibition experiments *in vitro* using cultured PBMCs. Thus, blockade of TNFα in supernatants of RA PBMCs added to PBMCs from HCs reduced the capacity of monocytes from the HCs to differentiate to pOCL [24]. These findings are in agreement with a previous study that reported reduction in pOCL after treatment with TNFα inhibitors [44]. This reduction was attributed to a reduction in RANK-L expression on lymphocytes and a general reduction in $CD14^{++}CD16^{-}$ monocyte numbers. Although classical

monocytes have relatively higher pOCL potential in HCs [45], it has been suggested that pOCLs may have an intermediate phenotype ($CD14^{++}CD16^{+}$) in inflammatory conditions [46]. This is important since there is a causal relationship between bone erosion and increased numbers and activity of osteoclasts [47]. In addition, treatment with TNFα inhibitors could reduce the generation of pOCL and bone loss through upregulating the recombinant recognition sequence binding protein at the J(κ) site (RBP-J) [48]. This transcription factor suppresses TNFα-induced osteoclastogenesis and bone loss by down regulating the expression of micro RNA 182 (miR-182) which is a positive inducer of the osteoclastogenic transcription factors NFATc1 and B lymphocyte-induced maturation protein-1 [49].

In conclusion, this study shows that TNFα blockade reduces bone loss in RA patients irrespective of patients' DAS28 clinical response. The data also shows that the beneficial bone response occurs, at least partly, through reduced osteoclastogenesis and IL-20 production. Reduction in BMD loss, however, occurs only in the lumbar spine but not in the hip of treated patients. This data may suggest that IL-17 plays a role in bone loss in the hip of RA patients [50]. Improvements in lumbar spine BMD significantly correlated with reduced levels of TNFα but not with changes in plasma bone biomarker levels. The study, however, has limitations. For example, the number of non-responder patients was relatively small. This impacts the statistical significance of the findings. Therefore, a study with a larger cohort of patients could consolidate these findings. In addition, findings from the study rely on statistical analyses as is the nature of clinical studies. Further direct studies targeting key cytokines and pro-osteoclastogenesis downstream of TNFα will be necessary to determine directly how TNFα blockade reduces generalized bone loss in RA. Furthermore, it remains unclear how does IL-17 contribute to generalized bone loss in RA patients and what the effects of TNFα and IL-17 are on osteoblasts.

Author contribution: **M. Al-Bogami and J. Bystrom**: Contributed to the conception of parts of the work and the acquisition, analysis and interpretation of the data and preparing a draft of the manuscript. **F. Clanchy, TE. Taher, P. Mangat, RO. Williams and AS. Jawad:** contributed to the conception and design of parts the work, interpretation of data and providing the final approval of the published version of the work. **RA Mageed:** conceived and designed the work, involved in the acquisition and analysis of the data, drafting the manuscript and approving the final submitted version.

All co-authors have read and approved the content of this article.

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Figure Legends

Figure 1. T cell cytokine profiles of RA anti-TNFα responder and non-responder patients before treatment. T cells were enriched by negative selection from the blood of RA patients immediately before treatment. The cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (10 µg/mL) for 48 hrs and levels of cytokines produced in the culture determined in the resulting supernatants using MSD multiplex kits. Levels of 16 cytokines were determined but only results of cytokines with consistent differences are presented. The data are presented as the mean ± standard error of the mean (SEM) from 46 responder and 21 non-responder RA patients. Differences between responders and non-responders were assessed using two-tailed Mann Whitney U test; * indicates $P<0.05$.

Figure 2. Treatment with TNFα inhibitors marginally modulates cytokine and bone biomarker levels. (A-C) Cytokines were measured in anti-CD3/CD28 stimulated T lymphocyte culture supernatants before treatment and at 1 and 3 months (1m and 3m, respectively) after treatment using multiplex kits. (D-G) Levels of RANK-L, CTX, OPG and osteocalcin were determined in plasma from the patients at the same time-points using ELISA. Data from responder patients are presented in white columns and non-responders in black. Blood from 46 responder patients and 21 non-responder patients were analysed. The data are presented as the mean ± SEM and compared using Wilcoxon matched-pairs signed rank test.

Figure 3. Bone biomarker and cytokine level correlations in RA patients pre- and post-treatment with TNFα inhibitors. Plasma and supernatant levels of bone biomarkers and cytokines were correlated before the start of treatment with TNFα inhibitors. There were various levels of correlations but consistent and clear correlations were between CTX (**A** and **D**), OPG (**B** and **E**) and osteocalcin (**C**) and TNFα and IL-6 produced by culture supernatants

from activated T lymphocytes. The results are from analysing blood from 53 patients prior to the start of treatment. The data were analysed using Spearman rank correlation.

Figure 4. TNFα blockade reduces osteoclastogenesis in RA. The frequency of $CD11b^{+}CD14^{+}$ monocytes in blood and pOCLs in cultured PBMCs of RA patients were measured before and at 1 month and 3 months after treatment. The frequency of $CD11b^{+}CD14^{+}$ (**A**) and $RANK^{+}$ expression (**B**) on these monocytes were determined by FACS using PBMCs separated on Ficoll-Paque. Numbers of pOCLs in PBMCs, before and after treatment, were determined in PBMCs cultured for 14 days as described in the Methods section of cultured cells stained with tartrate-resistant acid phosphatase (TRAP) and confirmed as multi-nucleated under bright-field microscopy (**C**-**F**). Data in **A**, **B**, **E** and **F** are presented as columns of the mean ± SEM for from 8 patients either prior to treatment or at 1 and then 3 months (1m and 3m, respectively) after treatment and from 8 healthy controls. The data were analysed using Student's t test and significant differences indicated as * indicating $P<0.05$ or ** indicating $P<0.01$.

Figure 5. Supernatants from RA PBMCs significantly enhance the differentiation of HC monocytes to pOCLs. PBMCs from 6 healthy controls were cultured for 14 days with culture supernatants collected from cultured RA PBMCs, mixed 50:50 with fresh medium. (**A**) A microscopic view of PBMCs from a HC cultured for 14 days with medium alone and stained with TRAP. (**B**) PBMCs from the same HC cultured with 100 ng/mL recombinant RANK-L and 25 ng/mL human M-CSF. (**C**) Shows that culture supernatants from PBMCs from RA patients promote the differentiation of PBMCs from HCs to $TRAP^{+}$ cells. (**D**) Blocking TNFα in RA PBMCs' culture supernatants with infliximab reduced the number of $TRAP^{+}$ cell to below the normal differentiation of the HC cells. (**E**) Blocking TNFα in RA PBMNC supernatant with infliximab and OPG had a more pronounced effect on $TRAP^{+}$ cells from the

HCs than infliximab alone. The data are presented as the mean ± SEM from 3 independent experiments and 6 samples from HCs. The data were analysed using Student's t test and significant differences are indicated as * indicating $P<0.05$.

Supplementary Figure 1. Pro-inflammatory cytokine levels produced by T cells from female RA patients stratified based on age. T cells were enriched by negative selection from the blood of RA patients immediately before treatment. The cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies for 48 hrs and cytokine levels in culture supernatants measured using MSD multiplex kits. The data are presented as the mean ± standard error of the mean (SEM) for treated female RA patients segregated on the basis of age for those years 50 years of age or younger (≤50) and those older than 50 years (>50) pre-treatment with TNFα inhibitors. Differences between the two age groups were assessed using two-tailed Mann Whitney U test.

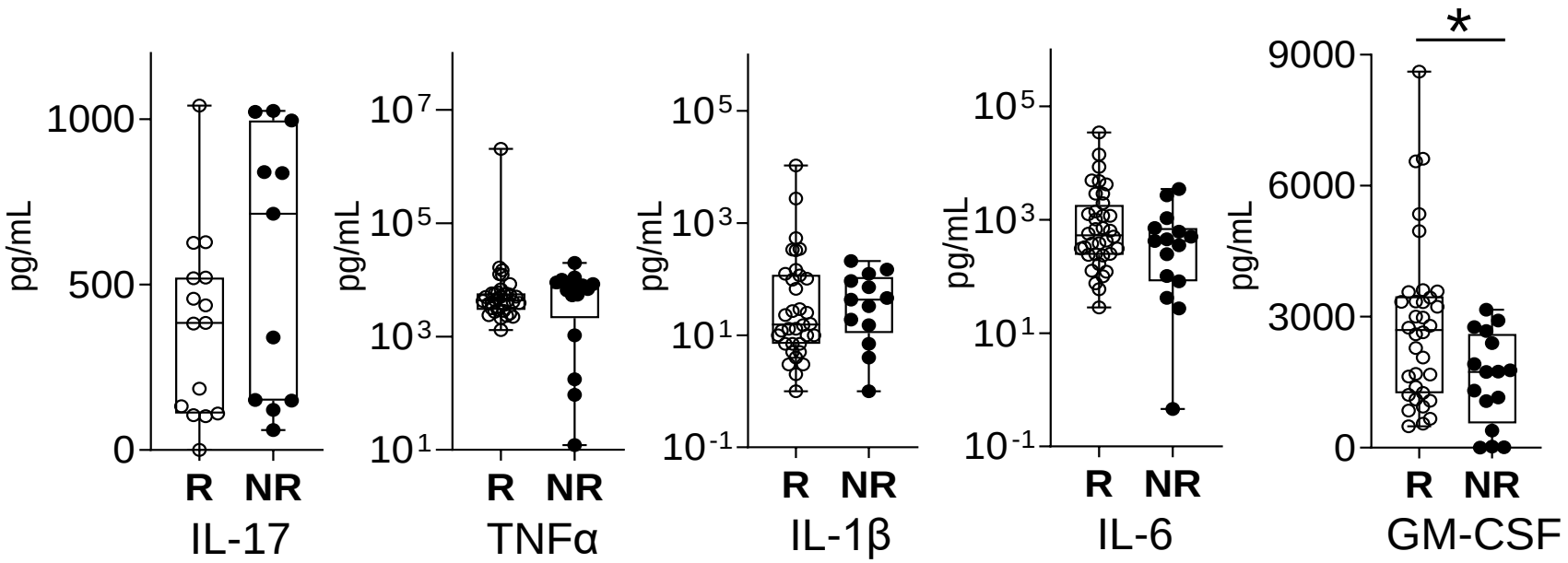
Supplementary Figure 2. Levels of IL-20 in RA patients correlate with TNFα levels in plasma and in supernatants of T cells cultured *in vitro*. Plasma levels of TNFα (**A**) and TNFα produced by cultured T cells (**B**) correlated with plasma levels of IL-20. Levels of IL-20 were determined by ELISA using specific monoclonal antibodies. The data are from 19 non-responder RA compared using Spearman rank correlation.

TABLE 1: Changes in BMD in RA patients treated with biologic TNFα inhibitors 2 years after treatment.

	Before treatment (mean±SD)	**2 years after treatment (mean±SD)**	***P***
BMD of lumbar spine in all RA patients (g/cm^2)	0.917±0.14	0.945±0.15	< 0.0001
- In responder patients	0.935±0.15	0.962±0.16	< 0.0001
- In non-responder patients	0.885±0.10	0.914±0.12	0.0733
BMD of hip in all RA patients (g/cm^2)	0.835±0.13	0.829±0.13	0.2
- In responder patients	0.853±0.15	0.851±0.14	0.81
- In non-responder patients	0.798±0.09	0.785±0.10	0.04

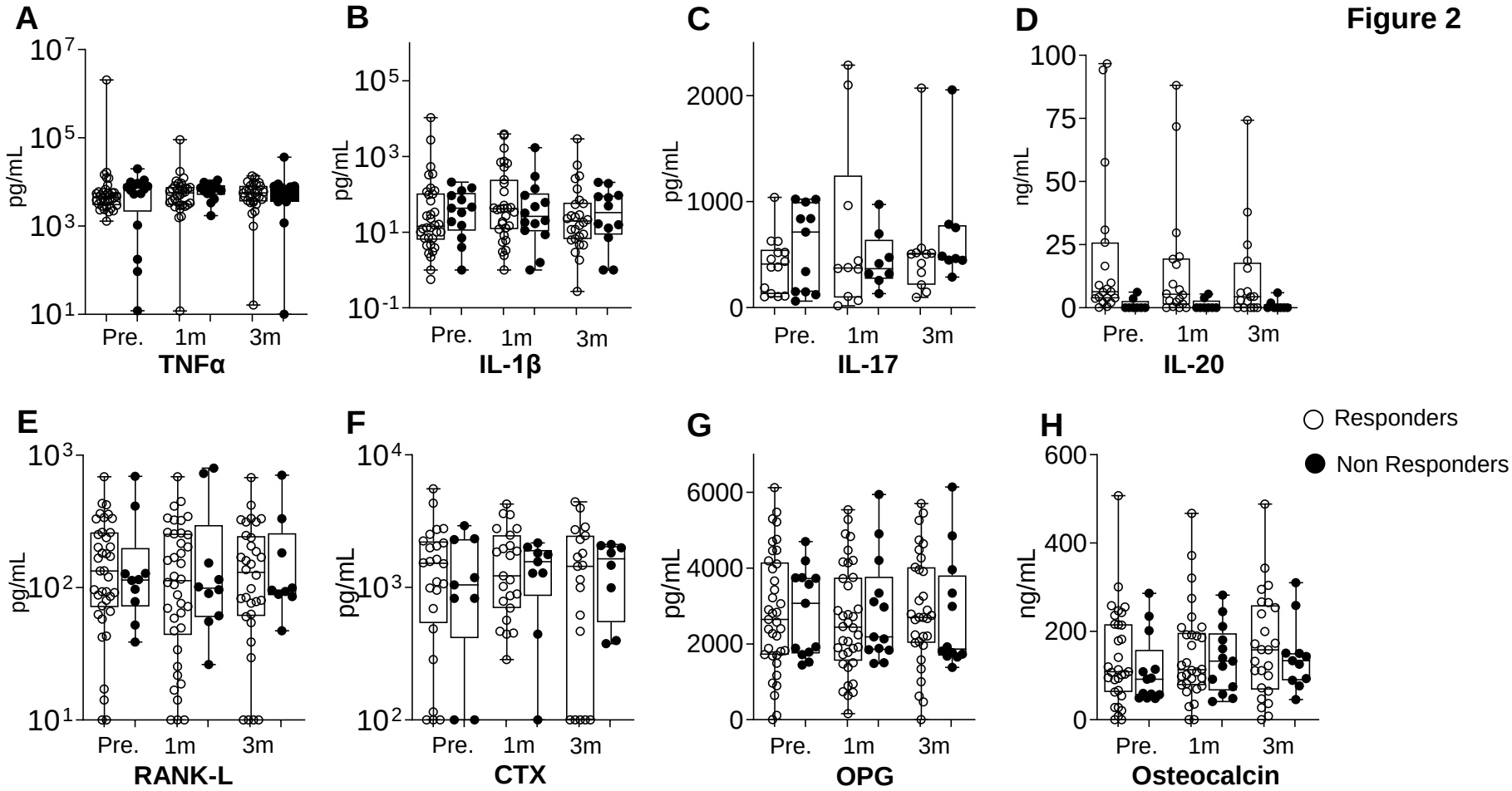
Changes in BMD as measured by DEXA scan in the cohort of 117 RA patients treated with biologic TNFα inhibitors. The patients were classified as responders or non-responders based on changes in DAS28. The response to treatment was based on the EULAR response criteria [25]. Patients were classified as responders when their DAS28 decreased by ≥1.2-3.2 after 3 months of treatment.

Figure 1



R: Responders

NR: Non Responders


A
B
C
D
Figure 2
pg/mL
pg/mL
pg/mL
ng/mL
Pre.
1m
3m
TNFα
IL-1β
IL-17
IL-20
E
F
G
H
Responders
Non Responders
pg/mL
pg/mL
pg/mL
ng/mL
RANK-L
CTX
OPG
Osteocalcin

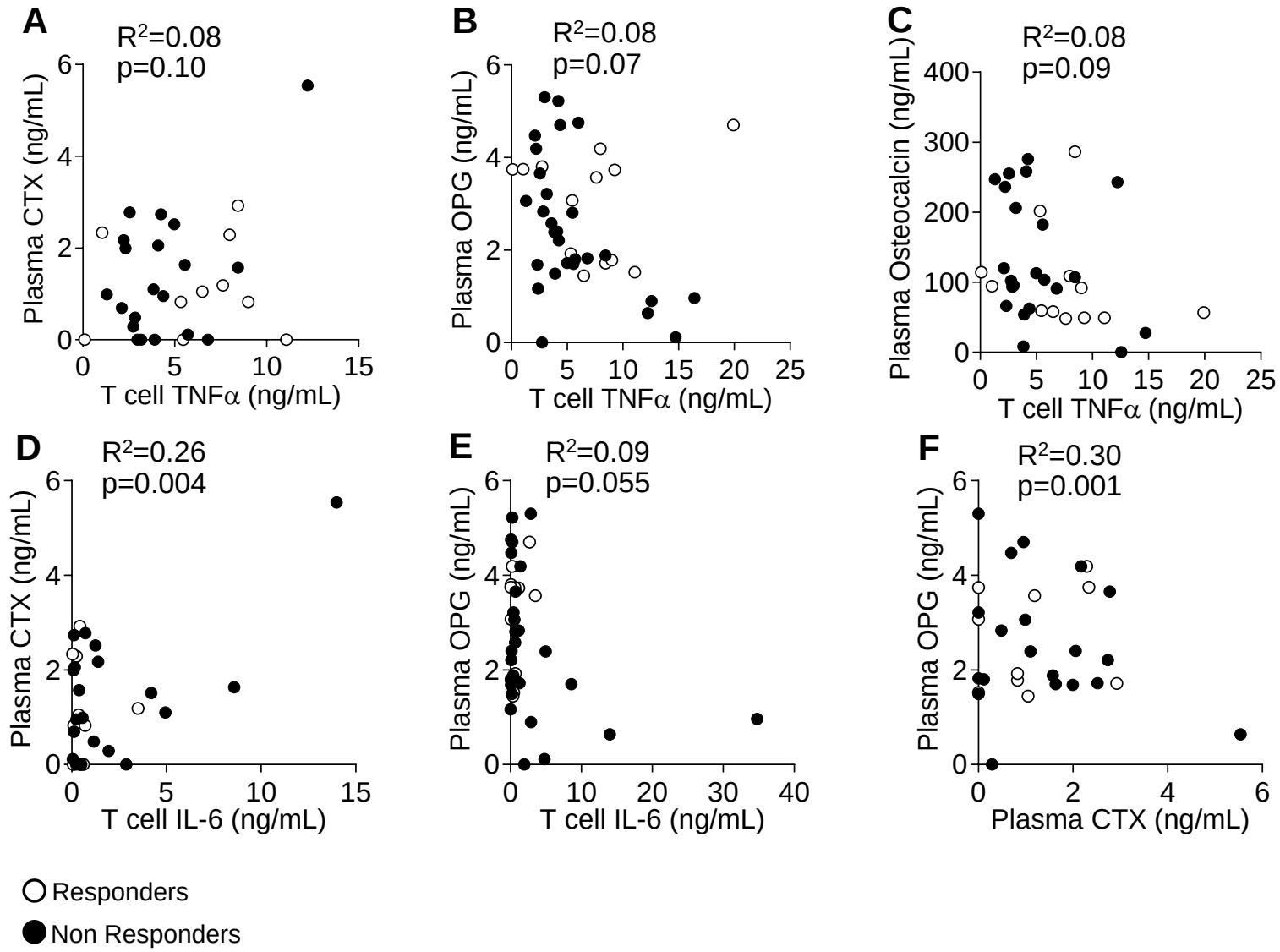

A
R²=0.08
p=0.10
Plasma CTX (ng/mL)
T cell TNFα (ng/mL)
B
R²=0.08
p=0.07
Plasma OPG (ng/mL)
T cell TNFα (ng/mL)
C
R²=0.08
p=0.09
Plasma Osteocalcin (ng/mL)
T cell TNFα (ng/mL)
D
R²=0.26
p=0.004
Plasma CTX (ng/mL)
T cell IL-6 (ng/mL)
E
R²=0.09
p=0.055
Plasma OPG (ng/mL)
T cell IL-6 (ng/mL)
F
R²=0.30
p=0.001
Plasma OPG (ng/mL)
Plasma CTX (ng/mL)
Responders
Non Responders

Figure 3

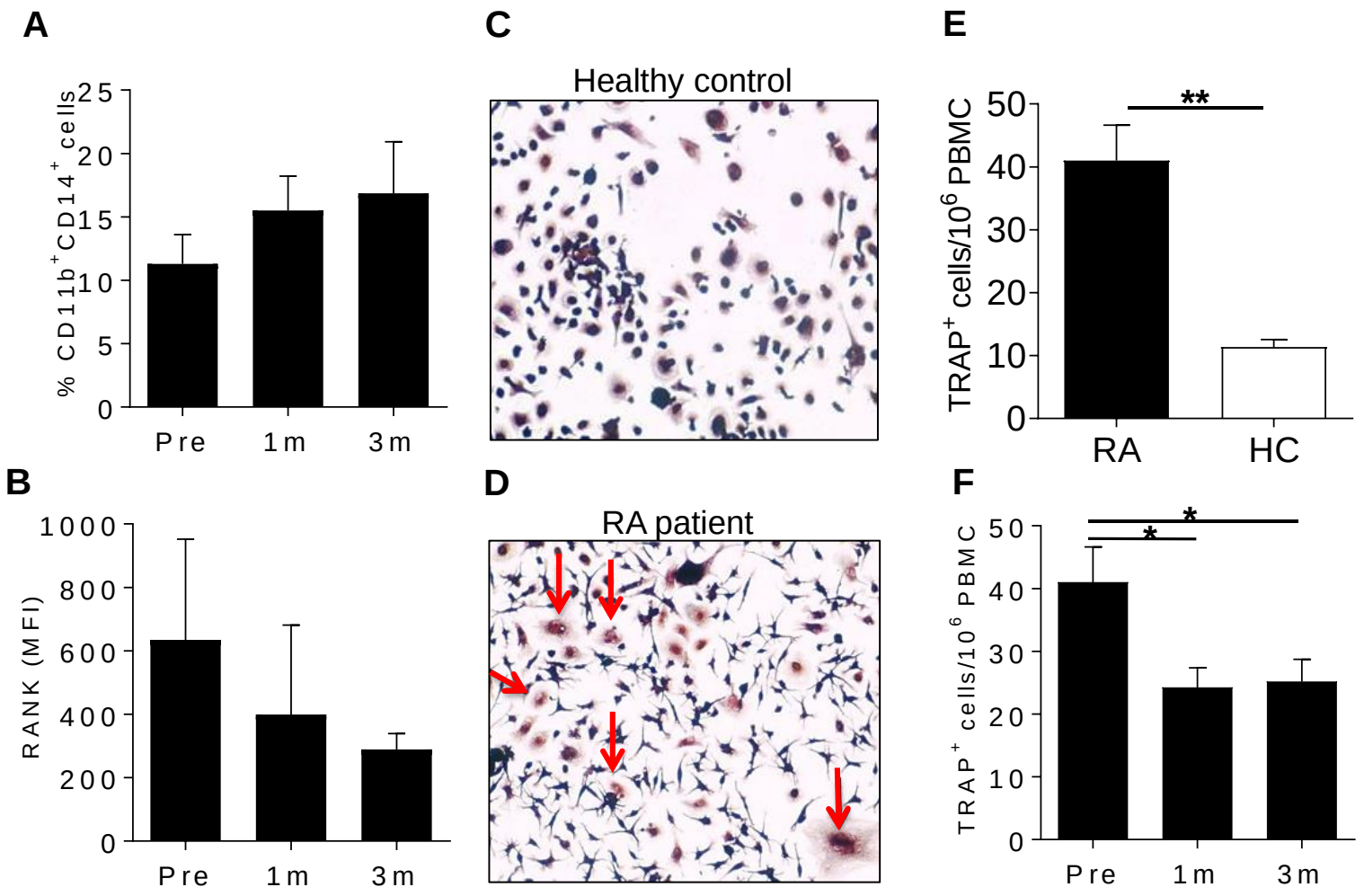

A
% CD11b+CD14+ cells
25
20
15
10
5
0
Pre
1m
3m
B
RANK (MFI)
1000
800
600
400
200
0
Pre
1m
3m
C
Healthy control
D
RA patient
E
TRAP+ cells/10^6 PBMC
50
40
30
20
10
0
**
RA
HC
F
TRAP+ cells/10^6 PBMC
50
40
30
20
10
0
*
*
Pre
1m
3m

Figure 4

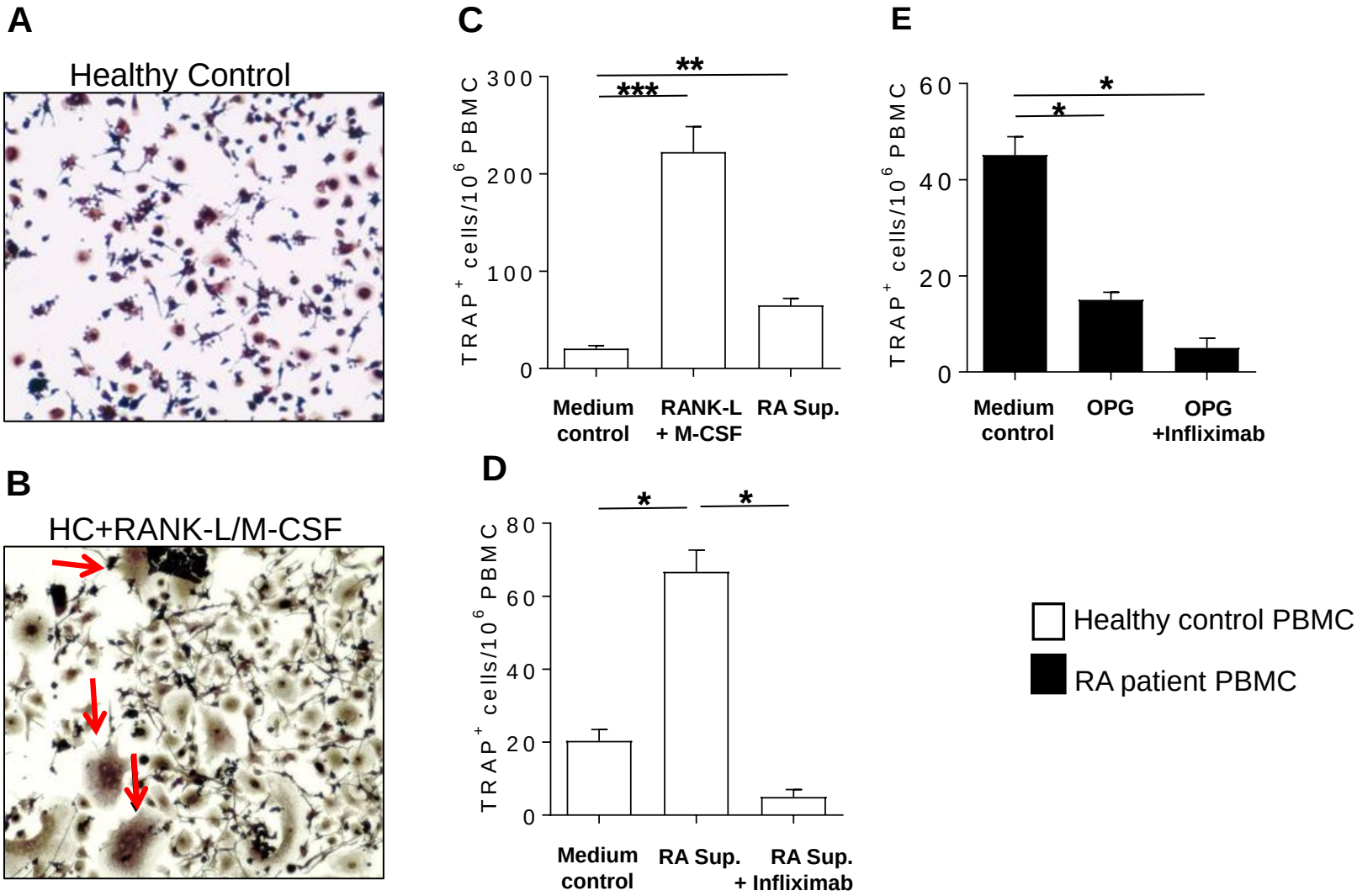
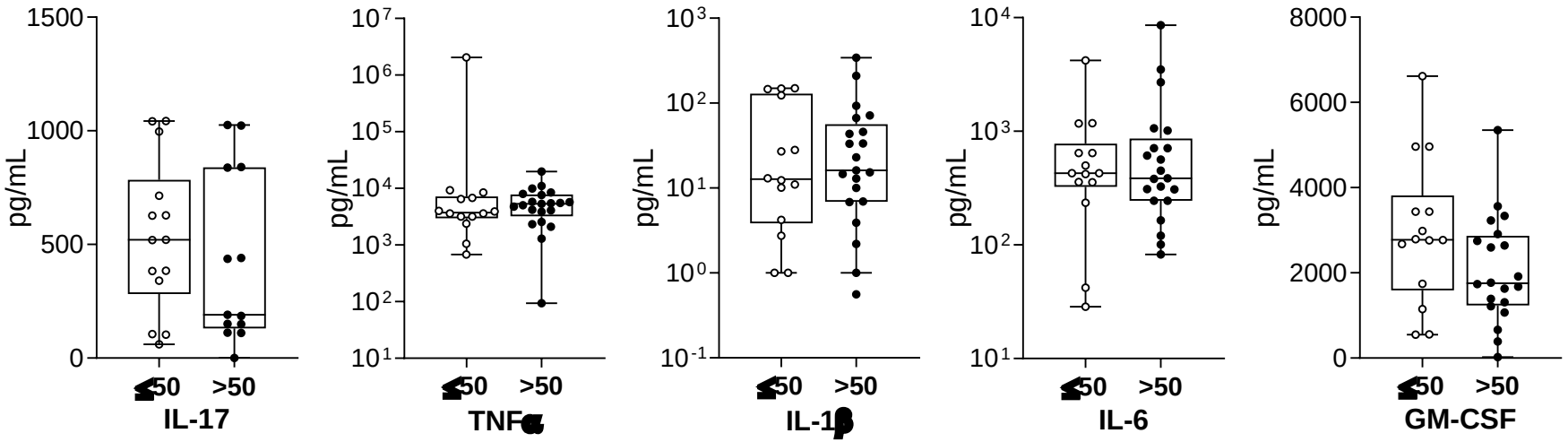
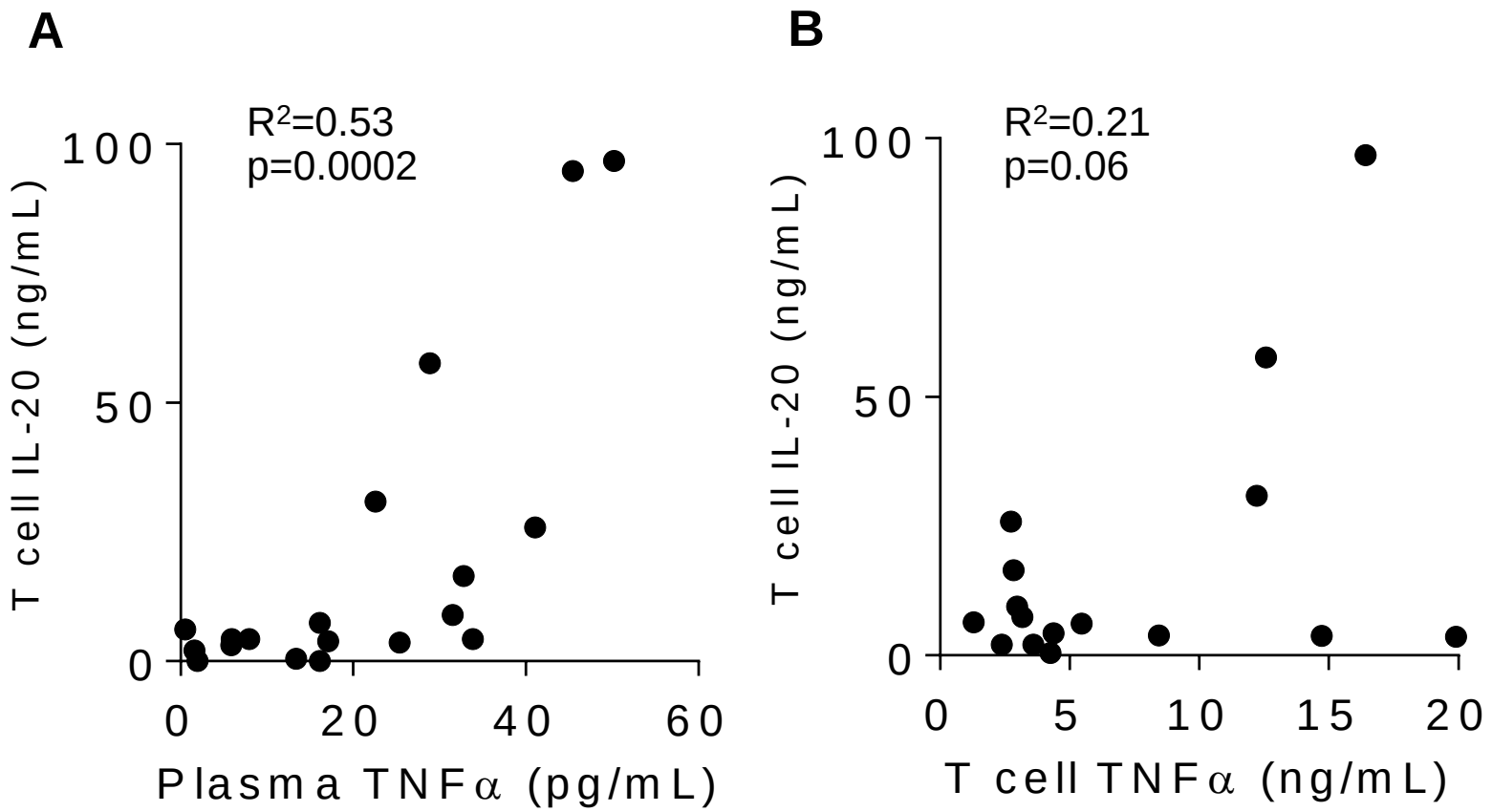

A
Healthy Control
B
HC+RANK-L/M-CSF
C
TRAP+ cells/10^6 PBMC
300
200
100
0
**
***
Medium control
RANK-L + M-CSF
RA Sup.
D
TRAP+ cells/10^6 PBMC
80
60
40
20
0
*
*
Medium control
RA Sup.
RA Sup. + Infliximab
E
TRAP+ cells/10^6 PBMC
60
40
20
0
*
*
Medium control
OPG
OPG +Infliximab
Healthy control PBMC
RA patient PBMC

Figure 5

Supplementary Figure 1



Supplementary Figure 2



Methods

Assessment of the immune system in treated patients

T cells, B cells and monocytes were enriched by negative selection using RosetteSep kits (STEMCELL Technologies, Cambridge, UK) [23]. The enriched cells were studied by FACS for membrane protein expression and intracellular cytokine production. Some of the cells were stimulated *in vitro* and cultured. T cells were cultured in the presence of 10µg/mL anti-CD3 mAb (clone OKT3) pre-coated onto culture plates and 10µg/mL anti-CD28 (clone 28.2) in solution. B cells were stimulated with 10µg/mL goat $F(ab')_2$ anti-IgM pre-coated onto culture plate wells and 10µg/mL anti-CD40 (clone G28-5) in solution. Monocytes were stimulated with 1µg/mL lipopolysaccharide (LPS). Culture supernatants were collected after 48 hrs and used for cytokine measurements. For intracellular cytokine production, T cells were stimulated with 0.1µg/mL PMA and Ionomycin in the presence of 1µl/mL Golgiplug™ (BD Bioscience) overnight and intracellular cytokine production measured using fluorochrome-conjugated mAbs with specificity for IL-17, TNFα or GM-CSF and analysed by FACS using a BD-LSR Fortessa X20.

Measurement of the frequency of precursors of osteoclast (pOCL)

Peripheral blood mononuclear cells (PBMCs) from 8 patients and 8 matched healthy controls (HCs) were separated on Ficoll-Paque, cultured *in vitro* and stained with tartrate-resistant acid phosphatase (TRAP; Sigma) to determine the frequency of pOCL. In addition, PBMCs were suspended in RPMI containing 10% FCS and penicillin/streptomycin. 10^6 PBMCs/mL were seeded into each well of 24-well plates and incubated at 37°C with 6% CO_2 in the air for 14 days. 50% of supernatants in each well were removed every 3 days and replaced with fresh medium. Collected supernatants from each individual patient, harvested every 3 days were pooled after 14 days, centrifuged and stored at -80°C until used. The differentiation of cultured PBMCs to pOCL was determined by counting the number of TRAP+ multinucleated cells. As

positive controls for pOCL differentiation, PBMCs from 4 HCs were cultured in parallel in the presence of 25ng/mL recombinant human M-CSF and 100ng/mL recombinant RANK-L. To assess the effect of RA disease on the differentiation of pOCL, supernatants collected from cultured PBMCs of RA patients as described above were mixed 50:50 with fresh medium and 2 mL of the mixture added to $2x10^5$ cultured PBMCs from each of 4 HCs. One mL supernatant was replaced with one mL of fresh RA PBMCs' supernatant mixed 50:50 with fresh medium every 3 days. The cells were maintained for 14 days and pOCL counted with TRAP staining as above. To determine the effect of blocking TNFα in the RA supernatants, 2.5μg/mL infliximab (Merck & Co) was added to parallel cultures of the HC PBMCs with the RA supernatants. At the end of the cultures, adherent cells were stained with TRAP and examined under a light microscope [24]. TRAP+ multinucleated cells, each containing ≥3 nuclei were enumerated and presented as pOCL/10^6 PBMCs.

Measurements of cytokines and blood bone biomarkers

For cytokine quantification in plasma and in culture supernatants of immune cells, multiplex MSD kits (MSD Technologies) were used. The following cytokines/chemokines were quantified in plasma and supernatants of stimulated T cells, B cells and monocytes with MSD kits: TNFα, IL-17, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-23p40, GM-CSF, IFNγ and MCP-1. IL-22 levels were measured by ELISA using DuoSet Abs (R&D Systems). Plasma RANK-L, OPG and IL-20 levels were quantified using ELISA kits (R&D Technologies). Plasma osteocalcin and C-telopeptide cross-links of collagen type I (CTX-I) were determined using ELISA kits (Immunodiagnostic Systems).