



## Programmed death ligand 2 – A link between inflammation and bone loss in rheumatoid arthritis

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### ABSTRACT

**Objective:** Active rheumatoid arthritis (RA) is accompanied by increased appendicular and axial bone loss, closely associated to the degree of inflammation. The programmed death-1 (PD-1) pathway is important for maintaining peripheral tolerance, and its ligand PD-L2 has recently been associated with bone morphogenetic protein activity. Here, we report that PD-L2 plays a central role in RA osteoimmunology.

**Methods:** Femoral bone mineral density (BMD) and trabecular bone microstructure were evaluated by micro-CT in wild type (WT) and PD-L2<sup>-/-</sup> mice. Osteoclasts were generated from RA synovial fluid mononuclear cells and peripheral blood monocytes. The effects of recombinant PD-L2, was evaluated by tartrate-resistant acid phosphatase (TRAP) activity and the development of bone erosions in the presence of anti-citrullinated protein antibodies (ACPA). Plasma soluble (s)PD-L2 levels were measured in patients with early (e)RA (n = 103) treated with methotrexate alone or in combination with the TNF inhibitor Adalimumab.

**Results:** PD-L2<sup>-/-</sup> mice had a decreased BMD and deteriorated trabecular bone microstructure that was not related to the RANKL/OPG pathway. PD-L2 decreased TRAP activity in osteoclasts and decreased ACPA-induced erosions. In the RA synovial membrane PD-L2 was highly expressed especially in the lining layer and plasma sPD-L2 levels were increased in eRA patients and decreased with treatment. One-year sPD-L2 correlated inversely with erosive progression two years after treatment initiation with methotrexate and placebo.

**Conclusion:** PD-L2 regulates bone homeostasis in RA. Our findings provide new insight into the relationship between the immune system and bone homeostasis, and suggest a potential therapeutic target for limiting inflammatory bone loss in RA.

### 1. Introduction

The immune system is a central regulator of bone homeostasis [1].

This close relation is exemplified in inflammatory conditions, where bone turnover is altered, resulting in decreased bone mass and increased risk of fractures and physical disability [2,3].

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Clinically detectable inflammation in rheumatoid arthritis (RA) is initially located to the joints, where the balance between bone formation and resorption is shifted towards increased degradation by the presence of pro-inflammatory cytokines. The chronic inflammation will cause an imbalance in bone remodeling, which results in a more general loss of bone mass accompanied by an increased fracture risk [4–6]. The inflammation in RA is driven by an imbalanced immune system, where decreased tolerance towards self-antigens is a key element [7,8]. The presence of autoantibodies, especially anti-citrullinated protein antibodies (ACPA), is associated with an increased risk of joint destruction [9]. Moreover, it has been shown that purified ACPAs increased osteoclastic erosions *in vitro* [10]. Citrullination of peptides carried out by the peptidylarginine deiminase (PAD) enzymes is also essential for osteoclast activity [11,12]. Finally, cells within the synovium, including fibroblast-like synoviocytes (FLS), have been shown to play a major role in the joint destruction. Among others, FLS produce Receptor activator of nuclear factor kappa  $\beta$  ligand (RANKL) thus enhancing the ongoing osteoclastogenesis [13,14].

T cell activation is carefully restricted by several immune checkpoints. Programmed death-1 (PD-1) is an important co-inhibitory receptor with two known ligands; PD-L1 and PD-L2 [15], and this pathway is impaired in RA [16,17]. Both PD-1 and its ligands are present in soluble (s) forms [18], which are upregulated in RA and other inflammatory conditions [16,19–22]. Soluble PD-L2 is cleaved from the cell surface [23] as a bioactive molecule [18]. It remains unclear whether members of the PD-1 pathway affect bone homeostasis, like other mediators of immune regulation; Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) and regulatory T cells that have previously been shown to limit bone loss [24–26]. However, a recent study demonstrates the relation between treatment with immune checkpoint inhibitors, including anti-PD-1 and increased risk of fractures [27].

A novel receptor complex for PD-L2 consisting of repulsive guidance molecule b (RGMb) or DRAGON, in conjunction with bone morphogenetic protein (BMP) and neogenin has been identified [28]. Neogenin is also identified as a receptor for BMPs, regulating signal transduction and affecting bone homeostasis [29].

In the present study, we tested the hypothesis that the PD-L2 pathway is associated with bone homeostasis in RA, influencing osteoclastogenesis and osteoclast function.

## 2. Methods

### 2.1. Patients and healthy controls

Early RA (eRA) patients from the Optimized Treatment Algorithm in Early Rheumatoid Arthritis (OPERA) cohort were randomly selected for this study ( $n = 103$ ). The OPERA study is described in detail elsewhere [30]. In brief, treatment naïve eRA patients with an average disease duration of 3 months were randomly assigned to two groups. All patients were treated with corticosteroid injections in swollen joints and the conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) methotrexate (MTX). In addition, one group was treated with the tumor necrosis factor inhibitor (TNFi) adalimumab (ADA), while the other group received placebo (PLA) (Table 1). After 12 months ADA/PLA was discontinued, and patients were followed for an additional 12 months on csDMARDs. Radiographs of hands and feet were obtained at baseline and after 12 and 24 months.

Plasma and synovial fluid samples were obtained from chronic RA (cRA) patients ( $n = 31$ ) with more than 8 years of disease, and were collected when the patients presented with disease flare. All received different DMARDs and some also a biological agent. Healthy controls (HC,  $n = 38$ ), from the Danish blood bank, with a similar distribution of age and gender as the OPERA cohort were included as controls. For tissue staining, sections were obtained from cRA patients ( $n = 3$ ) undergoing surgery for joint replacement. No additional clinical data were available from these patients.

**Table 1**

**Patient characteristics at baseline.** Data are presented as median with 5–95 percentile. Mann-Whitney U or Chi<sup>2</sup> test was used to calculate the differences between the two groups. CRP: C-reactive protein; ACPA: anti-CCP antibodies; DAS28CRP: disease activity score in 28 joints including CRP; SDAI simplified disease activity index; CDAI: clinical disease activity index; SJC: swollen joint count; TJC: tender joint count both evaluated in 28 and 40 joints.

	DMARD + ADA	DMARD + PLA	P value
No of patients	52	51	
Age	52 (26–77)	54.4 (29–80)	0.61
Gender (% female)	59.6	64.8	0.59
IgM-RF + (%)	67.3	82.4	0.079
ACPA + (%)	59.6	78.4	0.049
CRP	14 (6–105)	14.5 (7–116)	0.69
DAS28CRP	5.3 (3.6–7)	5.4 (3.6–7.5)	0.52
TJC28	10 (3–19)	10.3 $\pm$ 4.9	0.83
SJC28	7 (1–19)	9 (3–28)	0.39
TJC40	15 (4–29)	14.5 (3–38)	0.43
SJC40	10 (2–28)	9 (3–30)	0.98
SDAI	31.0 (12–55)	30.7 (14–65)	0.45
CDAI	28.6 (11–47)	29.2 (11–63)	0.50
Patients with radiographic changes at baseline (measured by TSS) (%)	19.2	26.0	0.41
Days since diagnosis	90 (42–152)	86.5 (42–160)	0.94

### 2.2. Mice

Male C57BL/6 knockout mice PD-L2<sup>-/-</sup> ( $n = 4$ ) and PD-L1/L2<sup>-/-</sup> ( $n = 4$ ) and WT mice (Taconic, USA) were kept in Scantainers under controlled conditions (21–25 °C, 30–60% humidity and 12-h light/dark cycle). At eight weeks, mice were euthanized and the blood was collected and centrifuged at 1600 rpm for 10 min to isolate the serum, which was kept at –80 °C until further analysis. The femur was carefully removed, cleaned, and kept at –80 °C.

### 2.3. Bone DXA scan and serum bone parameters

The left femur was pDXA scanned (Sabre XL, Norland Stratec) at a pixel size of 0.1  $\times$  0.1 mm<sup>2</sup>. Bone mineral content (BMC) and area bone mineral density (aBMD) of the whole femur were determined [31].

RANKL and osteoprotegerin (OPG) were measured in serum using commercially available ELISA kits (ab100749 and ab100733, Abcam), in accordance with the manufacturer's instructions.

### 2.4. Micro CT

The left distal femoral metaphysis was  $\mu$ CT scanned (Scanco  $\mu$ CT 35, Scanco Medical AG, Brüttisellen, Switzerland) with 1000 projections/180°, an isotropic voxel size of 3.5  $\mu$ m, an X-ray tube voltage of 55 kV<sub>p</sub> and current of 145  $\mu$ A, and an integration time of 800 ms. A 1000- $\mu$ m-high volume of interest (VOI) starting 300  $\mu$ m above the most proximal part of the growth zone containing trabecular bone only was demarcated. Similarly, an 819- $\mu$ m-high mid-diaphyseal VOI was  $\mu$ CT scanned with 500 projections/180°, an isotropic voxel size of 7  $\mu$ m, and integration time of 300 ms. A cortical VOI was demarcated with the contour tool of the scanner software, thus delineating the periosteal bone surface. The data were Gaussian filtered ( $\sigma = 0.8$ , support = 1), threshold filtered (533.8 mg HA/cm<sup>3</sup>) and analyzed [32]. 3D visualization was made using Amira 5.6 (FEI Visualization Science Group, Mérégnac, France).

## 2.5. Osteoclast generation and function

Osteoclasts were generated from HC peripheral blood mononuclear cells (PBMCs) and cRA synovial fluid mononuclear cells (SFMCs). After every 3 days medium was supplemented with: (25 ng/ml) rhM-CSF, (50 ng/ml) rhRANKL, as previously described [33]. Both HC and SFMC cultures were stimulated with rhPD-L2 his tag (800 ng/ml) (10292-H08H, Sino Biological) when medium was changed. All cultures were analyzed after 21 days. Tartrate resistant acid phosphatase (TRAP) activity was assessed in all osteoclast cultures using a commercially available TRAP staining kit (PMC-AK04F-COS, B-Bridge International, Inc). Bone erosion assay was performed as previously described: In short, monocytes were differentiated as above along with 10 µg/ml polyclonal ACPA or the control IgG [11]. Osteoclasts were generated on a synthetic calcium phosphate surface (Corning, Amsterdam, Netherlands) for 14 days and the eroded surface area was quantified using NIS-elements (Nikon, Tokyo, Japan).

## 2.6. PAD2 and PAD4 mRNA expression in SFMCs

RA PBMCs and SFMCs were stimulated with either 10 ng/ml TNF $\alpha$  (Peprotec), 800 ng/ml rhPD-L2/B7-DC Fc chimera protein (R&D Systems), or 800 ng/ml Mouse IgG1 Isotype control (R&D Systems) for 2 h at 37 °C and 5% CO $_2$  at a cell density of 1–2.5  $\times$  10 $^6$  cells/ml.

RNA was extracted using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. The concentration of RNA was determined using the Nanodrop 1000 Spectrophotometer. Followed by real-time PCR analysis using the Taqman RNA to Ct 1-step kit (Applied Bioscience) and the following TaqMan assays: PAD-2 (Hs00247108\_m1), PAD-4 (Hs00202612\_m1), and HPRT1 (Hs02800695\_m1) (all from ThermoFisher Scientific). The relative amount of PAD-2 and PAD-4 mRNA were calculated using the formula: 2 $^{-\Delta Cq}$  with (Hypoxanthine-guanine-phosphoribosyl-transferase 1) HPRT1 as the reference gene, and normalized to the N/A sample (not-activated) to obtain a relative ratio.

## 2.7. Fibroblast-like synovial cells

FLS were grown from SFMCs from cRA patients as previously described [34]. FLS at passage 4–5 were cultured in a 24-well plate, stimulated with TNF- $\alpha$  (20 ng/ml), and interferon gamma (IFN- $\gamma$ ) (10 ng/ml) for 72 h and stained for flow cytometry using: PE conjugated anti-RANKL (clone: M1H24, Biolegend), PC7 conjugated anti-PD-L1 (clone: PDL1.3.1, Beckman Coulter), APC conjugated anti-PD-L2 (clone: M1H18, BD Pharmingen) and FITC conjugated anti-CD90 (clone: 5E10, BD Pharmingen). Unspecific binding was blocked with 100 µg/ml mouse IgG prior to staining [35].

## 2.8. Osteoblast mineralization

Human osteoblasts (C-12720, Promocell) were cultured in osteoblast growth medium (C-27010, Promocell) (n = 4, repeated twice). Cells were seeded at 20,000 cells/well. When confluent, rhPD-L2 (800 ng/ml) was added to the culture medium. BMP2 and osteoblasts were cultured for a total of 21 days. The mineralization was visualized using the OsteoImage Bone Mineralization Assay (PA-1503, Lonza), in accordance with the manufacturer's instructions. Imaging was performed by fluorescence microscopy (Leica, DM IRBE).

## 2.9. Soluble PD-L2 ELISA

Plasma sPD-L2 was measured in eRA, cRA, and HC samples using a commercially available ELISA kit (sPD-L2: DY1224, R&D systems) including heterophilic blocking [36]. In eRA patients, plasma levels were investigated for correlation with C-reactive protein (CRP), presence of IgM-Rheumatoid Factor (IgM-RF), presence of ACPA, progression of structural joint damage on radiographs evaluated by delta total Sharp

Score ( $\Delta$ TSS) [37], and clinical disease activity markers; including disease activity score in 28 joints/CRP (DAS28CRP), simplified disease activity index (SDAI), clinical disease activity index (CDAI), swollen joint count (SJC), and tender joint count (TJC) evaluated in both 28 and 40 joints.

## 2.10. Immunohistochemical staining

Whole tissue sections acquired from RA metacarpophalangeal (MCP) joints, during joint replacement, were cut from formalin fixed, paraffin embedded tissue blocks, deparaffinized in xylene, and rehydrated in an ethanol gradient. Staining was carried out by anti-PD-L2 clone 3G2 (Merck Research Laboratories, Palo Alto CA) [38].

## 2.11. Study approval

Human clinical studies were conducted in accordance with the Helsinki declaration. All patients provided written informed content to participate in the study. Studies were approved by the Danish Data Protection Agency (2007-41-0072), the Danish Medical Agency (2612-3393), and the Regional Ethics Committee (1-10-72-82-15 and 2012-1329-2). Plasma from anonymous HCs was obtained from an established cooperation with the Danish blood bank. All mice were used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Health Guidelines.

## 2.12. Statistics

Statistical analyses were performed using Stata 13 (Stata Nordic, Sweden) and Graphpad Prism (Graphpad Software, CA, USA). Results for sPD-L2 were log-transformed in order to fit the normal distribution. Differences in sPD-L2 levels were analyzed using Student's *t*-test. Spearman ranked correlation was used to investigate associations between sPD-L2 levels and clinical or laboratory variables. All correlation analyses were adjusted for smoking status, disease duration in days, gender, and age. Other data sets were analyzed using parametric statistics when applicable, and non-parametric statistics when data did not meet the criteria for the normal distribution. In all cases, *p* < 0.05 was considered statistically significant.

## 3. Results

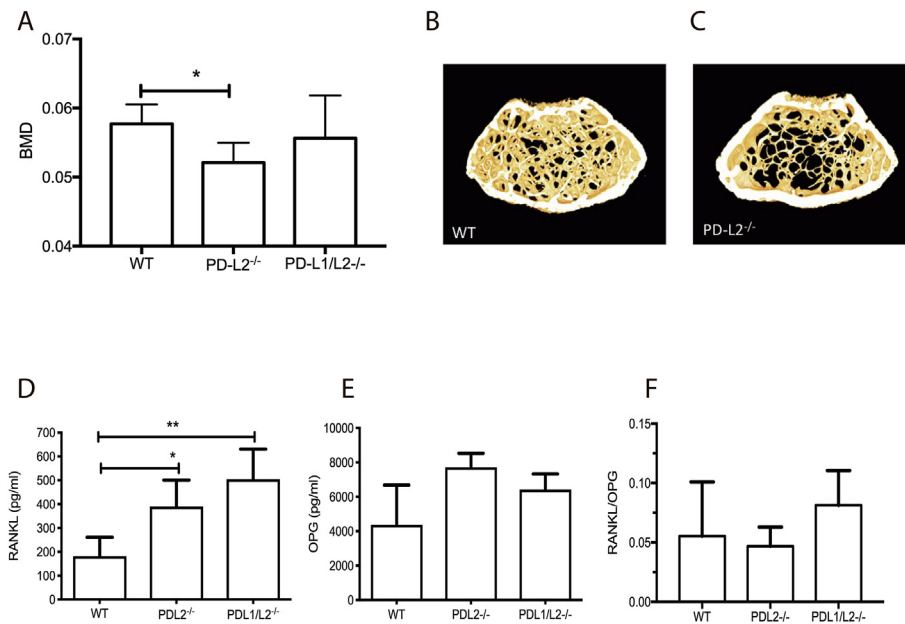
### 3.1. PD-L2 plays a central role in bone homeostasis

We examined the effects of PD-L2 on bone homeostasis in a non-inflammatory model. Areal BMD of 8-week-old PD-L2 $^{-/-}$  C57BL/6 mice was significantly lower than in WT mice, whereas the areal BMD of PD-L1/L2 $^{-/-}$  mice did not differ from that of WT mice (Fig. 1A). The decreased femoral areal BMD was confirmed by a subsequent  $\mu$ CT scan, revealing a significant decrease in both trabecular and cortical bone parameters in PD-L2 $^{-/-}$  mice, whereas this was not the case for PD-L1/L2 $^{-/-}$  mice (*p* < 0.05) (Fig. 1B, C and Fig. 2A and B). RANKL was significantly higher in both PD-L2 $^{-/-}$  and PD-L1/L2 $^{-/-}$  mice than in WT mice (Fig. 1D). However, OPG was also higher, resulting in an unchanged RANKL/OPG ratio (Fig. 1E and F). This suggests that in the non-inflammatory condition the RANKL/OPG axis does not play a significant role in the PD-L2-induced changes in bone homeostasis.

### 3.2. PD-L2 influences osteoclastogenesis in RA

Osteoclasts were generated from HC monocytes and RA SFMCs. SFMCs provides an inflammatory and *ex vivo* model for generating RA osteoclasts.

Both HC monocytes and SFMCs were stimulated with rhPD-L2-Ig fusion protein alone, and in combination with rhRANKL and rhM-CSF. The addition of rhPD-L2 decreased TRAP activity significantly in



**Fig. 1. BMD is decreased in PD-L2<sup>-/-</sup> mice.** (A) Femoral bone mineral density (BMD) evaluated by DXA of WT, PD-L2<sup>-/-</sup>, and PD-L1/L2<sup>-/-</sup> mice. BMD was significantly lower in PD-L2<sup>-/-</sup> mice compared with WT mice. (B) and (C) Representative  $\mu$ CT images through the distal femoral metaphysis from a WT and a PD-L2<sup>-/-</sup> mouse. (D) RANKL serum levels in WT, PD-L2<sup>-/-</sup>, and PD-L1<sup>-/-</sup>PD-L2<sup>-/-</sup> mice. Serum RANKL levels in WT mice were significantly lower than in knockout mice. (E) OPG serum levels in WT, PD-L2<sup>-/-</sup>, PD-L1<sup>-/-</sup>, and PD-L1<sup>-/-</sup>PD-L2<sup>-/-</sup> mice. Serum OPG levels did not differ between WT and knockout mice. (F) The ratio between serum RANKL and OPG in WT, PD-L2<sup>-/-</sup>, and PD-L1<sup>-/-</sup>PD-L2<sup>-/-</sup> mice. The ratio did not differ between the mice. (n = 4 in each group). Differences between the groups are evaluated by one-way ANOVA with subsequent *t*-tests. Bars represent mean with SD. Asterisk (\*) represents  $p < 0.05$  and \*\* represents  $p < 0.01$ .

rhRANKL and rhM-CSF stimulated cultures. Most pronounced in the SFMC cultures (Fig. 3A and B).

In multi nuclei osteoclasts cultures, PD-L2 was mainly expressed on the cell surface and its receptor RGMB was predominantly present intracellularly (Fig. 3C and D).

We examined PD-L2's ability to influence ACPA-driven bone erosion. PD-L2 directly inhibits ACPA-induced osteoclastic erosions on synthetic calcium phosphate-coated plates ( $p < 0.005$ ) (Fig. 3E). PD-L2 did, however, not influence SFMC or PBMC synthesis of the major mediators of citrullination in RA; PAD-2 (Fig. 3F) and PAD-4 (data not shown).

Finally, PD-L2 did not influence bone homeostasis through osteoblasts, as the addition of rhPD-L2 to osteoblast cultures did not influence mineralization (data not shown).

### 3.3. IFN- $\gamma$ induces PD-L2 on RANKL expressing FLS

PD-L2 expression on non-stimulated FLS was low (<5%), however, stimulating with IFN- $\gamma$  or TNF- $\alpha$  increased PD-L2 expression significantly (Fig. 3G). RANKL was expressed by ~5% of the FLS. Addition of IFN- $\gamma$  or TNF- $\alpha$  increased PD-L2 surface expression especially on the RANKL<sup>+</sup> FLS (Fig. 3H).

### 3.4. Soluble PD-L2 is increased and associated with disease progression in early RA

In accordance with immune activation, plasma levels of sPD-L2 were increased in eRA.

Both treatment regimens reduced sPD-L2 levels significantly, however only by treatment with MTX + ADA levels reached those of HCs (Fig. 4A and B).

We examined whether sPD-L2 plasma levels were associated with disease activity and inflammation. At baseline, sPD-L2 correlated linearly with CRP (slope: 0.027, CI: 0.00052–0.053,  $p = 0.046$ ). We did not observe additional associations to markers of disease activity, nor to ACPA or IgM-RF status. Neither did we observe correlation to disease activity at other time points. However, regarding radiographic progression, levels of sPD-L2 after one year correlated negatively with two-year radiographic progression ( $\Delta$ TSS) (Slope: -0.28, CI: -0.6; -0.05,  $p = 0.04$ ) in MTX + PLA treated eRA, whereas no correlation was present in the MTX + ADA treated group (Fig. 4C and D). The correlation was driven by the formation of erosions ( $\Delta$ Erosion score, Slope: -0.44, CI:

-0.85; -0.045,  $p = 0.03$ ), but not by joint space narrowing. Though plasma levels of sPD-L2 did not differ between ACPA positive and negative patients in the MTX + PLA patients, the association to  $\Delta$ TSS was only present among ACPA positive patients (Slope: -0.34, CI: -0.60; -0.068,  $p = 0.02$ ) and IgM-RF positive patients  $\Delta$ TSS (-0.35, CI: -0.61; -0.082,  $p = 0.012$ ) (Fig. 4E and F).

### 3.5. Soluble PD-L2 is not increased in chronic RA, but expressed by the synovium

Plasma sPD-L2 was lower in cRA patients than in HCs (Fig. 5A,  $p < 0.001$ ). In the paired plasma and synovial fluid samples, sPD-L2 levels did not differ, but were closely correlated (slope: 0.90, CI: 0.71; 1.1,  $p < 0.0001$ ) (Fig. 5B). Immunostaining of synovial tissue revealed PD-L2 primarily localized to cells in the lining layer (Fig. 5C), and in the sub-lining layer (Fig. 5D).

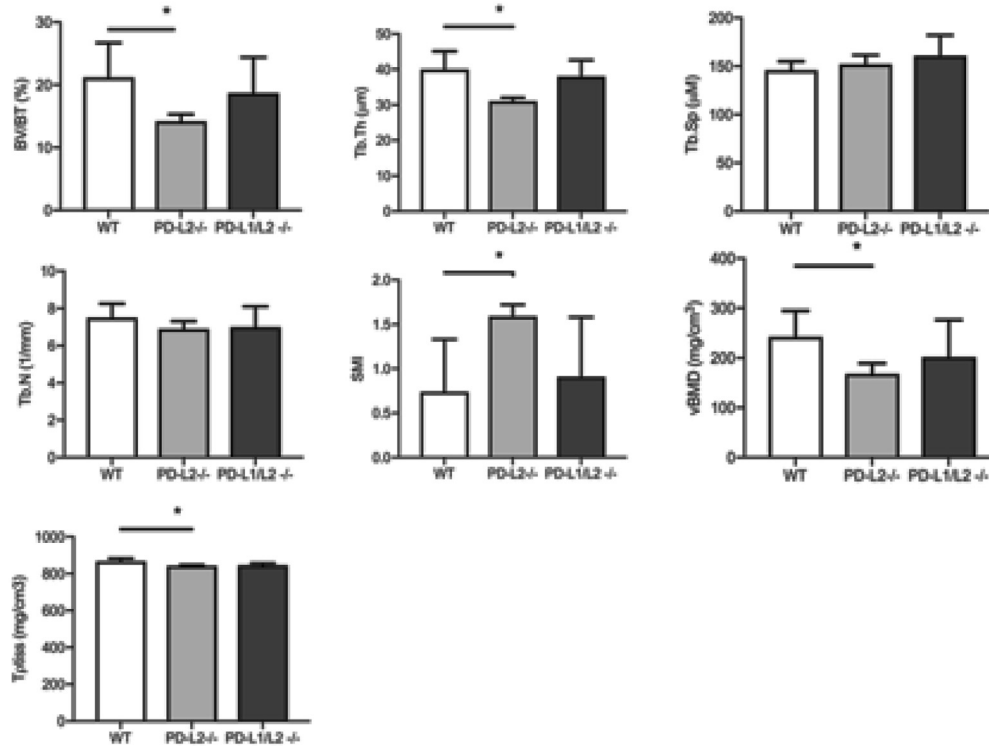
## 4. Discussion

Here, we report that PD-L2 is associated with bone loss during development and in osteoclastogenesis by reducing TRAP activity and inhibiting ACPA-dependent osteoclast activity. Finally, PD-L2 is also associated with less bone erosions in patients with newly diagnosed ACPA and IgM-RF positive RA. This suggests an important role for PD-L2 in regulating bone homeostasis during inflammation, and normal growth.

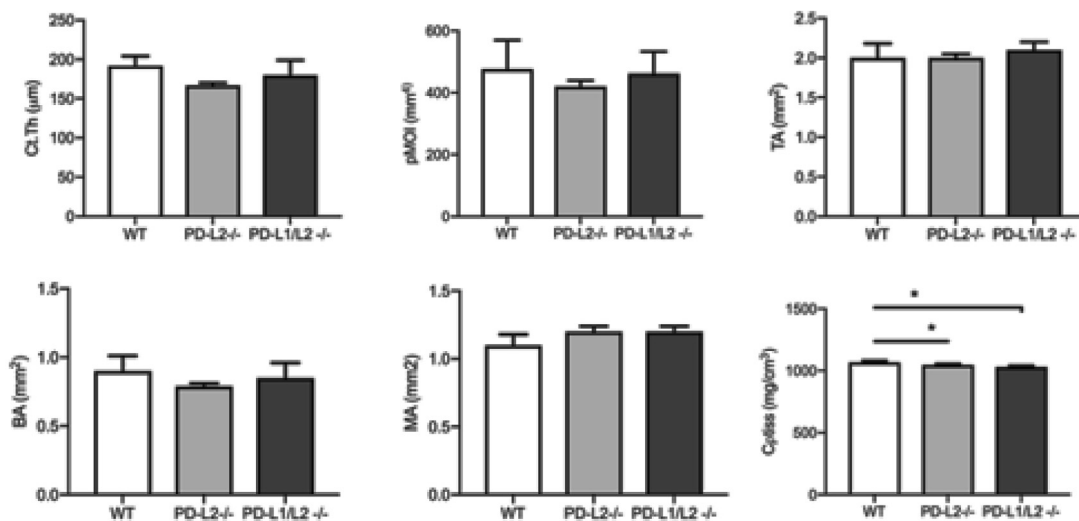
Recently, it was reported that patients undergoing treatment with immune checkpoint inhibitors (anti-PD-1 and anti-CTLA4) presented with increased risk of fractures [27]. This highly supports the importance of immune checkpoints in bone homeostasis in inflammatory conditions.

Presence of ACPA and IgM-RF is central to joint destruction in RA [39]. ACPA's ability to induce both osteoclastogenesis and bone resorption is presumably influenced by numerous factors [11]. We here report the identification of such a factor; PD-L2. PD-L2 does not influence ACPA induced osteoclast activity by changes in PAD2 or PAD4 synthesis, but acts in a more direct manner. PD-L2 directly inhibits induction of osteoclast formation and bone-resorptive activity induced by ACPA. In addition, PD-L2 also inhibits osteoclast formation and activity in the absence of ACPA, as reduced TRAP activity was observed in both RA SFMC cultures and HC PBMC cultures, when these were stimulated with RANKL and M-CSF. These *in vitro* studies support a direct inhibitory effect

A



B



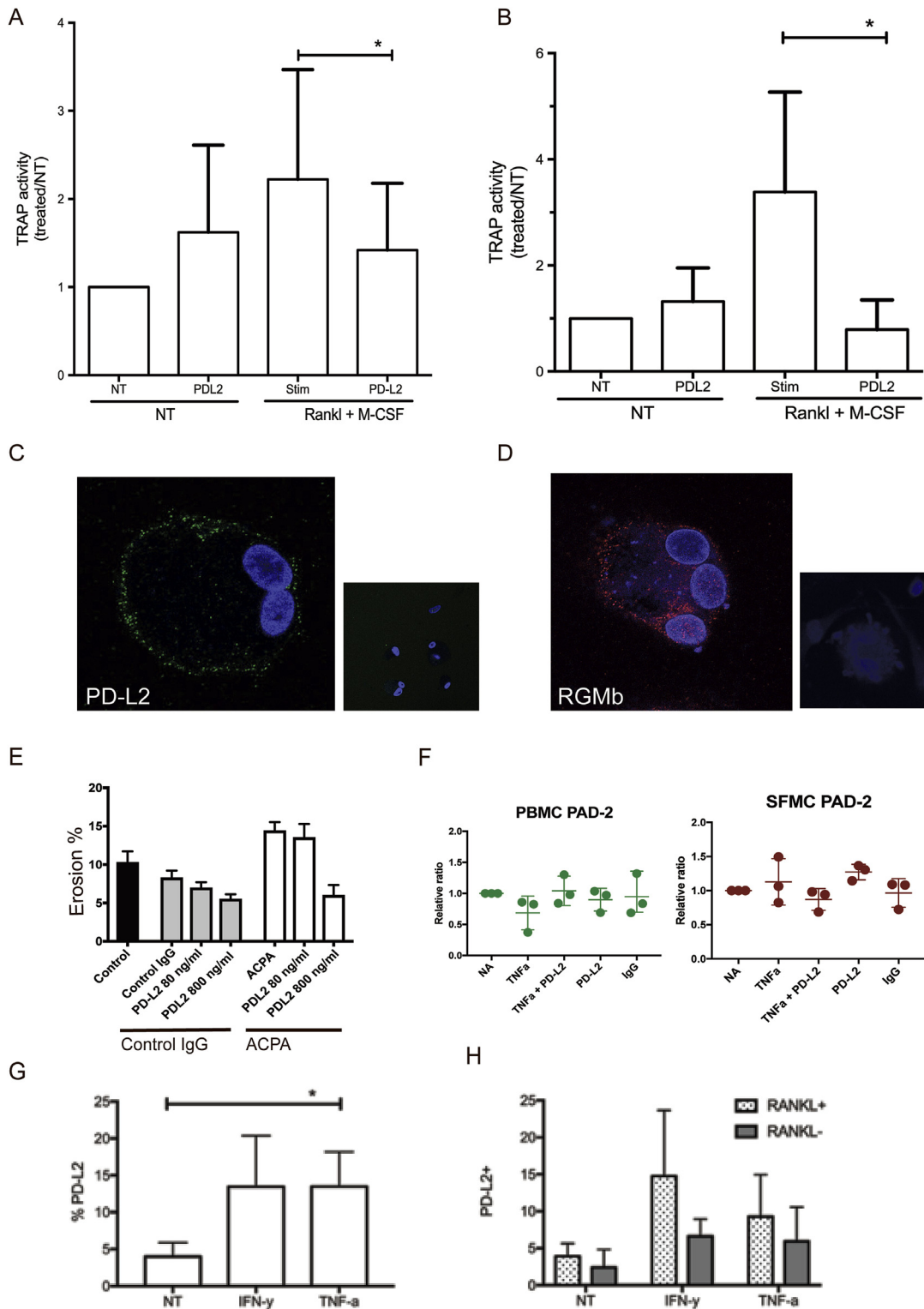
**Fig. 2.**  $\mu$ CT scanning of the femur from WT and PD-L2<sup>-/-</sup> and PD-L1/L2<sup>-/-</sup> mice. Data are presented as mean with SD. Differences are evaluated by Student's *t*-test ( $*p < 0.05$ ) or signed rank sum test ( $\#p < 0.05$ ). (A) Changes in trabecular bone in knockout mice. (B) Changes in cortical bone in knockout mice. PD-L2<sup>-/-</sup> mice especially differed from WT when evaluating trabecular bone. BV/TV [32]: bone volume/tissue volume; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; Tb.N: trabecular number; SMI: structure model index; vBMD: volumetric bone mineral density;  $\rho_{tiss}$ : bone tissue density, Ct.Th: cortical thickness, pMOI: Polar Moment of Inertia, TA: tissue area, BA: bone area, MA: marrow area, Cp.tiss: Cortical density. Asterisk (\*) represents  $p < 0.05$ .

on osteoclast activity by PD-L2.

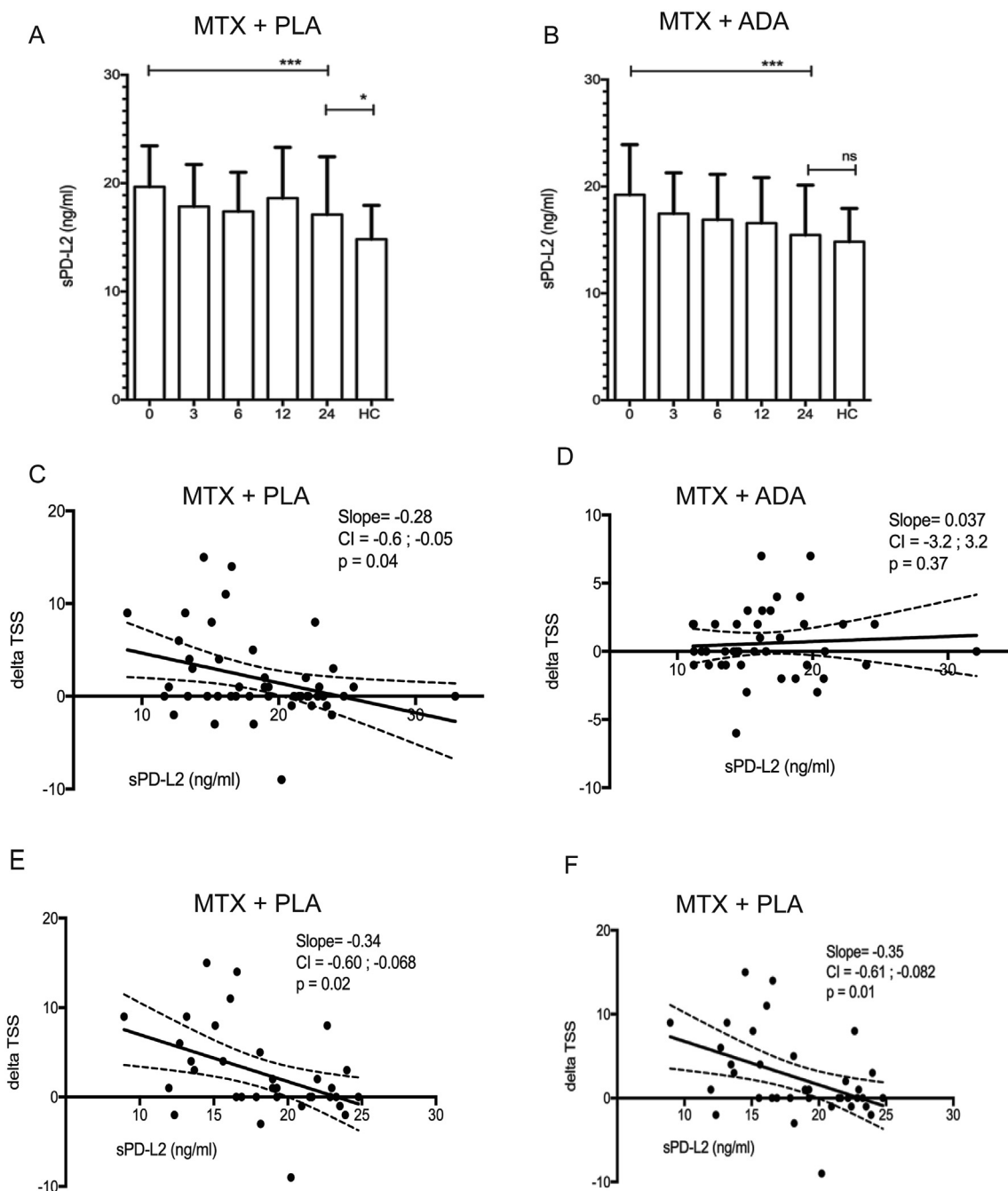
We and others have previously shown the PD-1 pathway to be associated with disease activity and outcome in eRA [16,21,40]. As seen for sPD-1, plasma levels of sPD-L2 were increased in treatment naïve eRA. However, at one year using a treat-to-target strategy, plasma sPD-L2 was inversely correlated with the formation of new erosions over 2 years. This was only seen in the MTX + PLA treated group among ACPA and IgM-RF positive patients, linking PD-L2, also in the clinical setting, to counteract central mechanisms of erosion in eRA. Due to random selection we, have

a little less ACPA positive patients in the MTX + ADA treated group. However, from our data, the association between delta TSS and sPD-L2 in the ADA treated group is not “near-significant”, and even if we only evaluate the ACPA + patients we do not see any association.

TNF- $\alpha$ , especially in combination with ACPA and IgM-RF, is a major inducer of osteoclastogenesis [41,42]. As seen with the emerging new treatments in RA, the progression of TSS in our clinical cohort was low, however still the MTX + ADA treatment did reduce radiographic erosive progression compared with MTX + PLA [43]. This is probably why



**Fig. 3. PD-L2 decreases TRAP activity in osteoclast cultures and its ligand RGMB is expressed by osteoclasts.** Osteoclast cultures were established from healthy controls (HC) (A), and from synovial fluid mononuclear cells from chronic rheumatoid arthritis joints (B) TRAP activity was measured after 21 days. TRAP activity was determined by the measured optical density (OD), and data are presented as the OD of stimulated cultures normalized by the OD of non-treated (NT) cultures. Addition of rhPD-L2 significantly decreased TRAP activity in cultures co-stimulated with rhRANKL and rhM-CSF ( $n = 3-13$  donors). Bars represent mean with SD. Asterisk (\*) represents  $p < 0.05$ . (C) Osteoclasts stained for the presence of PD-L2 (green) and a nuclear DAPI staining (blue) with matching isotype control. (D) Osteoclasts stained for the presence of RGMB (red) and a nuclear DAPI staining (blue) with matching isotype control ( $n = 3$ ). (E) PD-L2 decreased erosions induced by ACPA. Osteoclast activity evaluated by erosions on synthetic calcium phosphate coating plates ( $n = 4$ , replicates). (F) PAD2 mRNA levels in response to PD-L2 stimulation in PBMCs ( $n = 3$ ) and SFMCs ( $n = 3$ ) respectively. Levels were not affected by PD-L2 supplement. (G) Surface expression of PD-L2 on CD90<sup>+</sup> FLS, non-treated (NT) and stimulated with TNF- $\alpha$  and IFN- $\gamma$  (All  $n = 4$ ). Expression of PD-L2 increased by stimulation. (H) Surface expression of PD-L2 on CD90<sup>+</sup> FLS, gated on RANKL<sup>+</sup> and RANKL<sup>-</sup> FLS. Stimulation especially increased PD-L2 expression on RANKL<sup>+</sup> FLS. Bars represent mean with SD. Asterisk (\*) represents  $p < 0.05$  and asterisk (\*\*\*) represents  $p < 0.001$ .

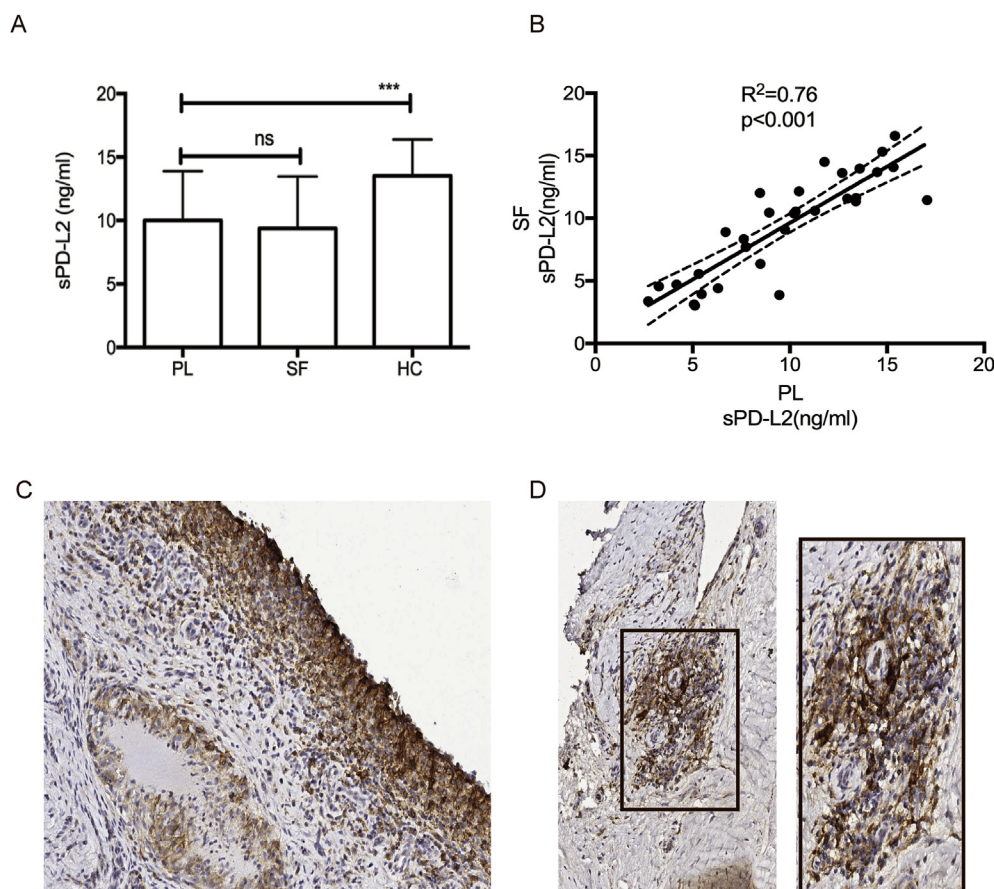


**Fig. 4. Increased plasma levels of sPD-L2 in eRA patients.** Plasma levels of sPD-L2 in patients with early rheumatoid arthritis, treated with methotrexate + placebo (MTX + PLA (n = 51) (A) or methotrexate + adalimumab (MTX + ADA) (n = 52) (B). In both treatment groups, plasma levels of sPD-L2 decreased significantly following treatment. After two years, sPD-L2 plasma levels were still significantly higher than in healthy controls (HCs) in the MTX + PLA group (A). No difference was observed in the MTX + ADA treated group (B). Bars represent mean with standard deviation (SD). C and D: Linear regression plots of sPD-L2 after one year and total Sharpe score (delta TSS) from baseline to two years in the MTX + PLA treated group (C) and the MTX + ADA treated group (D). E and F: Linear regression plots of sPD-L2 after one year and total Sharpe score (delta TSS) from baseline to two years in the MTX + PLA treated group for ACPA positive patients (E) and IgM-RF positive patients (F). The solid line represents the best-fitted line and the dashed lines represent the confidence interval. The significant difference refers to all data covered by the bar. Asterisk  $***p < 0.0001$ ,  $*p < 0.05$  and "ns": not significant.

plasma sPD-L2 only showed association with  $\Delta$ TSS in the MTX-PLA group. The MTX + ADA group reach levels of sPD-L2 in HC after two years of treatment, using a treat-to-target protocol. This is in line with the lower immune activity, also observed clinically [30]. Despite low levels of sPD-L2, radiographic progression in this group is less than the MTX + PLA group. Thus, for most patients, ADA treatment seems to be superior in protecting against structural damage compared to PD-L2. However, some patients still progress with erosions, and therefore new targets of

treatment are of continued interest. The association was restricted to ACPA or IgM-RF positive patients, which is in line with our *in vitro* studies, where PD-L2 decreased ACPA induced osteoclast activity. This further supports the central role of PD-L2 in influencing bone homeostasis in the inflammatory environment.

Uncoupling bone resorption and formation in RA is not restricted to osteoclast only. Immune resolution has also been shown to involve FLS that are high producers of RANKL. We show how FLS from the synovial



**Fig. 5. PD-L2 is present in the RA synovium.** (A) Levels of sPD-L2 in cRA patients in plasma (PL) and synovial fluid (SF) compared to plasma levels in healthy controls (HCs). Bars represent mean with standard deviation (SD). (B) Relationship between sPD-L2 in plasma (PL) and synovial fluid (SF). The relationship fitted a linear regression model. (C) and (D) Representative image of synovial membranes from chronic RA patients stained for the presence of PD-L2 (n = 3). Asterix \*\*\* $p < 0.0001$ , “ns”: not significant.

joint co-express PD-L2 with RANKL after stimulation with TNF- $\alpha$ , an increase similar to what is seen in the peripheral blood after immune activation [44]. This again supports a regulatory role for PD-L2 in the inflamed joint.

The question remains how PD-L2 influences bone homeostasis. Apart from binding to PD-1, PD-L2 also binds in a receptor complex with neogenin and RGMB, without interfering with BMP's binding to the same complex [28]. The expression of RGMB by osteoclasts and the decreased BMD by PD-L2<sup>-/-</sup>, whereas not by PD-L1/L2<sup>-/-</sup> mice support RGMB to be central.

Furthermore, PD-L2 in itself, did tend to induce osteoclast formation, although not significant. This is analogous with BMP-2 and -4 that both bind to RGMB and show the same changes of bone metabolism, linking PD-L2 and these BMPs and the same process in bone metabolism [45]. Taken together, these data support that PD-L2 regulates bone homeostasis by directly inhibiting osteoclastogenesis and ACPA-induced osteoclast activity.

Thus, our data suggest a prominent role for the co-inhibitory receptor PD-L2 in bone metabolism, especially in reducing osteoclast activity and limiting bone erosions in the inflammatory environment. This suggests that the PD-L2 may be a potential target for treating bone loss in RA, and potentially also in other inflammatory conditions.

#### Author contributions

Stinne Greisen: Conceptualization, Methodology, Formal analysis, Investigation, Writing, Original draft and review editing, project administration, funding acquisition. Tue Kragstrup: Conceptualization, Methodology, Writing review and editing, funding acquisition. Jesper Thomsen: Methodology, Formal analysis, Investigation, Writing review and editing. Aida Hansen: Methodology, Formal analysis, Investigation,

Writing review and editing. Akilan Krishnamurthy: Methodology, Formal analysis, Investigation. Kim Hørslev-Pedersen: Resources, Writing review and editing. Merete Hetland: Resources, Writing review and editing. Kristian Stengaard-Pedersen: Resources, Writing review and editing. Mikkel Østergaard: Resources, Writing review and editing. Lykke Midtbøll Ørnberg: Resources, Writing review and editing. Peter Junker: Resources, Writing review and editing. Arlene H. Sharpe: Supervision, Writing review and editing. Gordon J. Freeman: Supervision, Writing review and editing. Lakshmanan Annamalai: Methodology, Formal analysis, Investigation. Malene Hvid: Project administration, Supervision, Writing review and editing. Søren K. Moestrup: Supervision, Writing review and editing. Ellen-Margrethe Hauge: Writing review and editing, Investigation, Formal analysis. Anca Irinel Catrina: Writing review and editing, Investigation, Formal analysis. Bent Deleuran: Conceptualization, Supervision, Project administration, Funding acquisition, Writing review and editing. The final version of the manuscript was accepted by all authors.

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#### Data sharing statement

Additional data are available upon request.



## Patient and public involvement and ethical approval

Please refer to the methods section.

## Declaration of competing interest

Gordon Freeman and Arlene Sharpe will have disclosures. Otherwise authors have no disclosures or competing interests.

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## List of abbreviations

ACPA	of anti-citrullinated protein antibodies
ADA	adalimumab
aBMD	area bone mineral density
BMC	bone mineral content
BMD	bone mineral density
BMP	bone morphogenetic protein
CDAI	clinical disease activity index
CTLA-4	Cytotoxic T-Lymphocyte associated protein 4
cRA	Chronic rheumatoid arthritis
CRP	C-reactive protein
csDMARD	conventional synthetic disease-modifying anti-rheumatic drug
DAS28CRP	disease activity score in 28 joints/CRP
eRA	early rheumatoid arthritis
FLS	fibroblast-like synoviocytes
HC	Healthy controls
IFN- $\gamma$	interferon gamma
IgM-RF	IgM-Rheumatoid Factor
MCP	metacarpophalangeal
MTX	methotrexate
Opera	optimized treatment algorithm in early rheumatoid arthritis
OPG	osteoprotegerin
PAD	peptidylarginine deiminase
PBMCs	peripheral blood mononuclear cells
PD-1	programmed death 1
PD-L2	programmed death ligand 2
PLA	placebo
RA	rheumatoid arthritis
RANKL:	receptor activator of nuclear factor kappa $\beta$ ligand
RGMB	repulsive guidance molecule b
s	soluble
SDAI	simplified disease activity index
SFMCs	synovial fluid mononuclear cells
SJC	swollen joint count
TJC	tender joint count
TRAP	tartrate-resistant acid phosphatase
TNFi	tumor necrosis factor inhibitor
$\Delta$ TSS	delta total sharp score
WT	wild type

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