Osteoprotegerin in organ fibrosis: biomarker, actor, and target of therapy?
Putri, Kurnia Sari Setio

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CHAPTER 2

The RANK/RANKL/OPG axis has a role in regulating tissue repair processes in lung

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ABSTRACT

Osteoprotegerin (OPG) is associated with fibrotic processes, but its role in pulmonary fibrosis (PF) is unknown. OPG is a decoy receptor for Receptor-Activator-of-NF-κB Ligand (RANKL). RANKL can bind to RANK on macrophages, inducing degradation of extracellular matrix (ECM) in bone and OPG prevents this. Pulmonary macrophages also express RANK. We hypothesized that RANKL similarly induces ECM degradation in lung, while high levels of OPG dampen ECM degradation by macrophages and contribute to fibrosis development.

OPG levels were higher in fibrotic human and mouse lung tissue and correlated with higher collagen-1 content in mice. Expression was found in fibroblasts and isolated fibroblasts of PF patients had higher OPG production as compared to control fibroblasts. TGFβ stimulated OPG production in fibroblasts and in precision-cut-lung slices. RANKL-treatment of mice with fibrosis did not result in resolution but greatly induced OPG-production and epithelial cell numbers in lung.

In conclusion, the induction of OPG and the increase in epithelial cell numbers in lung following RANKL-treatment of fibrosis suggest that the RANK/RANKL/OPG-axis is not only active in bone, but also has a role in regulating tissue repair processes in lung. As OPG production is closely linked to fibrosis it is interesting new avenue to explore for drug and biomarker development.

Keywords: Pulmonary fibrosis, osteoprotegerin, resolution, macrophage and RANKL
INTRODUCTION

Pulmonary fibrosis is characterized by relentless scarring of the lung with extracellular matrix (ECM) deposition and progressive destruction of lung architecture that leads to impaired lung function with high mortality rates\(^1\,\!^{,}\,^{2}\). Fibroblasts and myofibroblasts are abundantly present in fibrotic lung tissue and are considered the main producers of excess ECM. Macrophages are also abundantly present in fibrotic lung tissue and can contribute to fibrosis by producing profibrotic cytokines\(^3\,\!,\,^{4}\). However, macrophages have also been shown to be antifibrotic by virtue of their ability to degrade ECM with matrix metalloproteinases (MMPs), cathepsins and their ability to internalize degraded collagens\(^5\,\!\!\!\!\,\!\!\!\!\,^{6}^{\text{-}}^{8}\). Gibbons et al. showed that resolution of experimental lung fibrosis in mice was attenuated when lung macrophages were depleted during the resolution phase of the disease\(^9\). However, it has not yet been elucidated whether this is a distinct population of macrophages and how their behavior is regulated.

OPG is a decoy receptor for receptor activator of nuclear factor κB ligand (RANKL), which is best known for its role in the regulation of bone ECM\(^10\). RANKL can stimulate macrophages in bone via its receptor RANK towards osteoclast differentiation and these can degrade bone ECM. As the decoy receptor for RANKL, OPG blocks the interaction of RANKL with RANK thereby inhibiting osteoclast differentiation and bone ECM degradation. Increased osteoprotegerin (OPG) expression has recently been associated with fibrotic processes in liver\(^10\,\!\!\!,\,^{11}\,\!\!\!,\,^{12}\) and heart\(^13\) and in the vasculature\(^14\). Increased OPG expression has also been observed in a mouse model of chronic lipopolysaccharide-induced airway remodelling\(^15\) and in silica- and bleomycin-induced lung fibrosis\(^15\). OPG levels increase early after bleomycin treatment and wane in time accompanied by a decrease in collagen deposition, suggesting that OPG may be important in active fibrotic processes. However, how OPG plays a role in fibrosis and whether it is involved in human pulmonary fibrosis remains to be determined.

As pulmonary macrophages were shown to express RANK as well (www.proteinatlas.com), we hypothesized that RANK activation could initiate a matrix resolving phenotype in the lung in the presence of RANKL, which may be hampered by high levels of OPG in fibrotic conditions. We therefore characterized the expression of
RANK, RANKL and OPG in lung tissue samples of patients with pulmonary fibrosis and of mice with experimental silica-induced lung fibrosis. To address the role of OPG in lung fibrosis, mice with silica-induced fibrosis were treated therapeutically with soluble RANKL (sRANKL) to neutralize OPG and we examined the progression of fibrosis in lung tissue.

MATERIALS AND METHODS

Human tissue

The study protocol was consistent with the Research Code of the University Medical Center Groningen, Dutch national ethical and professional guidelines, and the Medical Ethical Committee of Rotterdam. Patient characteristics are displayed in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Pulmonary Fibrosis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><strong>Cancer (n=15)</strong></td>
<td><strong>COPD (n=9)</strong></td>
</tr>
<tr>
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<td>FEV$_1$ % predicted</td>
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<tr>
<td>Diagnosis</td>
<td>3 lung cancer/3 lung metastases</td>
<td>1 GOLD stage 3/8 GOLD stage 4</td>
</tr>
</tbody>
</table>

Definition of abbreviations: PF = pulmonary fibrosis; FEV$_1$ = forced exhaled volume; COPD = chronic obstructive pulmonary disease; IPF = idiopathic pulmonary fibrosis; UIP = usual interstitial pneumonia.

Isolation and culture of primary fibroblasts

Primary lung fibroblasts were isolated from lung explants using a technique described previously$^{17,18}$. Patient characteristics are summarized in Table 2. Cells used in the experiments were maximally passaged three times. Plated cells were grown to confluence and transferred to low-serum medium (0.5% FCS). After 72 h, supernatants were collected for analysis.
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### Table 2. Characteristics of patients whose lung tissue was used for the isolation of primary lung fibroblasts (medians with range are presented)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Pulmonary Fibrosis</th>
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</thead>
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<td></td>
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<td>(n=6)</td>
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<td>2/1</td>
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<td>Age, years</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2 non-smoker/4 ex-smoker</td>
</tr>
<tr>
<td>FEV\textsubscript{1} % predicted</td>
<td>98 (90-130)</td>
<td>22.5 (20-25)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>Lung cancer</td>
<td>COPD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 IPF/UIP, 1 ILD/EAA, 1 NSIP/dust-exposed, 1 FNSIP/UIP</td>
</tr>
</tbody>
</table>

Definition of abbreviations: PF = pulmonary fibrosis; FEV\textsubscript{1} = forced exhaled volume; COPD = chronic obstructive pulmonary disease; IPF = idiopathic pulmonary fibrosis; UIP = usual interstitial pneumonia; ILD = interstitial lung disease; EAA = extrinsic allergic alveolitis; NSIP = non-specific interstitial pneumonia; FNSIP = fibrosing NSIP.

### Animal experiments

Male C57BL/6 mice were obtained from Harlan (Zeist, The Netherlands). All animal experiments were approved by the Institutional Animal Care and Use Committee, (DEC6064) and (DEC6416AA). Animal experiments were performed in the animal facility of the University of Groningen according to strict governmental and international guidelines on animal experimentation.

### Silica-induced lung fibrosis in mice

Fibrosis was induced using a single intratracheal dose of Min-U-Sil 5 crystalline silica (0.2 g/kg in 50 µl 0.9% saline, a kind gift from Dr. Andy Ghio, US EPA, Chapel Hill, NC) following isoflurane anesthesia. Control animals received an equivalent volume of 0.9% saline. Mice were sacrificed after 28 days and serum and lung tissue were collected (n=6-8).

In mice treated with sRANKL, silica was administered on day zero and sRANKL treatment began after 28 days and was continued for an additional 2 weeks. sRANKL was administered intranasally in 40 µl saline or 40 µl saline (vehicle) three times per week (n=12). On day 43, mice were sacrificed and lungs were collected for flow
cytometry, western blot analysis, ELISA, or histology. See **Figure 1** for an overview of the experimental setup.

**Figure 1.** Experimental setup of the animal experiments: pulmonary fibrosis was induced intratracheal (i.t.) instillation of silica on day 0. After 28 days, mice were either sacrificed to assess pulmonary fibrosis development or treated intranasally (i.n.) with sRANKL or vehicle for two additional weeks.

**Mouse lung precision-cut lung slices**

Lungs of six male C57BL/6 mice (20-30 gr, age 6-8 weeks) were used to make precision-cut lung slices. This method was previously described by Oenema et al.\(^{19}\). Lung slices were incubated in triplicate for 48h with TGF\(\beta\) (5 ng/ml) or 48h with TGF\(\beta\) followed by 24h of either vehicle or RANKL (200 ng/ml) stimulation. Three slices from each condition were pooled for further analyses and tissue slice supernatants were collected separately.

**Mouse cell cultures**

NIH 3T3 fibroblasts and RAW264.7 macrophages were purchased at the American Type Culture Collection. Self-propagating mouse alveolar-like macrophages (MPI cells) were a kind gift from Dr. G. Fejer, Plymouth University, Devon, UK. Before TGF\(\beta\) stimulation of 3T3 fibroblasts, cells were incubated in 0.5% FCS medium. After 24h, cells were stimulated with TGF\(\beta\) (5 ng/ml, Peprotech, Rocky Hill, NJ) for 48h after which the supernatant was collected.

To induce a proteolytic phenotype, RAW264.7 macrophages (until max. passage 12) or MPI macrophages were stimulated with 200 ng/ml soluble RANKL (sRANKL,
produced at our own facility). For each experiment, the data presented are representative of at least three independent replicates.

**Protein isolation from lung tissue**

Protein was isolated from frozen human or mouse lung tissue (30-40 mg) in buffer (0.25 M Tris/HCl buffer, 2.5 % Igepal, 0.5% SDS, Protease Inhibitor Cocktail (Boehringer Mannheim, Germany), pH 7.5). Overall protein concentration was determined using an RC DC™ Protein Assay based on the Lowry method (cat#5000111, Bio-Rad, Hercules, CA).

**Western blot analysis**

One hundred micrograms of tissue lysate per sample was used for western blot analysis. The following primary antibodies were used: mouse RANK (1:200, Acris, Herford, Germany), human RANK (1:100, R&D Systems, Minneapolis, MN), RANKL (human and mouse, 1:100, Acris) and β-actin (1:20,000 Sigma-Aldrich). RANK and RANKL expression levels were normalized to β-actin expression.

**ELISA**

Human and mouse OPG levels in lung tissue and culture supernatants were assessed using ELISA (cat#DY805 (human), cat#DY459 (mouse), R&D Systems) according to the instructions provided by the manufacturer. One hundred micrograms of tissue lysate total protein in a total of 100 μl was analyzed per sample. Cell culture media was analyzed undiluted while lung slice culture medium was diluted 1:5 before analysis.

**Immunohistochemistry**

Immunohistochemical analysis of collagen I, RANK, and OPG was performed on 3 μm paraffin sections of mouse lung tissue using anti-mouse collagen I (Southern Biotech, Birmingham, AL), anti-human RANK (R&D Systems), and anti-mouse OPG (Antibodies-online, Atlanta, GA). The amount of collagen deposition in the lung was determined using ImageScope software (Aperio, Burlingame, USA).
**Cathepsin K activity**

Cathepsin K activity of RAW264.7 macrophages was measured using a Cathepsin K Activity Kit (Cat#KA0769, Abnova, Taipei, Taiwan) following instructions provided by the manufacturer. Cathepsin K activity was normalized against the activity observed in unstimulated cells.

**Quantitative Real-time PCR**

Total mRNA was isolated from cells or tissues using a Maxwell® LEV simply RNA Cells/Tissue kit (cat# AS1280, Promega, Madison, WI) according to the instructions provided by the manufacturer. The following primers were used: collagen IαI, fibronectin, MMP9, OPG, β-actin, YWHAZ and 18s (Sigma-Aldrich, Zwijndrecht, The Netherlands). mRNA expression was normalized to β-actin for cell culture samples, to YWHAZ for tissue samples, and to 18S for precision-cut lung slices and expressed as fold-change of healthy controls or control conditions.

**Production and purification of sRANKL protein**

Recombinant sRANKL was produced by R. Cool following standard procedures at our facility.

**Flow cytometry**

Mouse-left lungs were used for the isolation of a single cell suspension for flow cytometry. Total cell numbers were determined using a FACS Array cell counter (BD Biosciences) and used to calculate total numbers of specific cell types. Single lung cell suspensions were stained for T cells, epithelial cells, neutrophils, interstitial and alveolar macrophage, and RANKL expression by flow cytometry. See supplemental Table 1 and 2 for antibody details. Samples were analyzed using an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (Tree Start, Ashland, USA). See supplemental data Figure 3 and 4 for the gating strategy.
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**Statistical analysis**

All groups were considered non-normally distributed due to small sample sizes. Statistical differences between two groups were assessed using Mann-Whitney U tests. Multiple-group comparison was performed with Kruskal Wallis. When significant, a Mann-Whitney-U test with a Bonferroni adjustment for multiple comparisons was used as a post-test. Correlations were assessed by calculating the Spearman correlation coefficient (R). Significance was considered when p<0.05. The data were analyzed using GraphPad Prism 6.

**RESULTS**

**OPG levels are higher in fibrotic lung tissue**

We observed higher OPG levels in human lung tissue with end stage fibrotic lung disease than in control lung tissue (Figure 2A). OPG levels in the lungs of silica-treated mice were also significantly higher when compared with control lungs (Figure 2B). Fibrosis in our mouse model was confirmed by significantly higher collagen expression and deposition in lungs of mice exposed to silica (Supplemental Figures 1A and 1B). Staining for OPG in mouse lung tissue showed that OPG expression localized in the smooth muscle layers around vessels and airways in control lung tissue (Figures 2C-D). In mouse lungs exposed to silica, OPG expression was also observed within areas of active fibrosis development, which was difficult to pinpoint to a specific cell type (Figures 2E-F, positive staining is indicated by the arrows). To assess whether OPG could be produced by fibroblasts in the fibrotic areas, human primary lung fibroblasts were tested for their ability to produce OPG. We found they produced copious amounts of OPG and lung fibroblasts from fibrotic lungs produced significantly more OPG than lung fibroblasts isolated from lung tissue of patients with lung carcinoma or COPD (Figure 2G). We also assessed whether bronchial or alveolar epithelial cells and macrophages could produce OPG and found that cultures of these cells did not produce any OPG (data not shown).
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A Human

B Mouse

C OPG (mouse healthy control)

D

E OPG (mouse experimental fibrosis)

F

G Primary human lung fibroblasts

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**Figure 2.** ELISA analysis of OPG levels on lung tissue lysates showed that OPG protein levels were higher in fibrotic conditions in both human (A) and mouse lung tissue (B). OPG levels were measured in control human lung tissue obtained from 15 patients undergoing surgical resection for carcinoma or COPD and pulmonary fibrosis tissue obtained from 11 patients undergoing lung transplantation. Mouse lung tissue was collected from mice 28 days after receiving silica (experimental fibrosis, n=7) or saline (healthy control, n=8). (C-D). Immunohistochemical staining for OPG in paraffin-embedded mouse lung tissue revealed expression in smooth muscle cells underneath endothelial cells and bronchial epithelial cells (E-F). Under fibrotic condition, OPG expression was also observed in fibrotic areas as indicated by the arrows, but it was unclear which cells were responsible for this staining. (G). Cell cultures of human primary fibroblasts isolated from lung tissue of patients with pulmonary fibrosis (n=6) or patients undergoing surgical resection for carcinoma or COPD (control patients, n=5) revealed that fibroblasts from fibrotic lung tissue produced significantly higher levels of OPG in culture supernatant than fibroblasts from control lung tissue. Groups were compared using a Mann-Whitney U test and p<0.05 was considered significant.

![Western blot analysis of RANKL](Image)

**Figure 3.** (A) Western blot analysis of RANKL shows expression of this protein in human lung tissue. RANKL levels were similar in lung tissue lysates from control patients undergoing surgical resection for carcinoma or COPD (n=15) and pulmonary fibrosis patients (n=11). (B) Western blot analysis of RANKL shows expression of this protein in murine lung tissue. Mouse lung tissue of healthy controls and mice with silica-induced pulmonary fibrosis showed similar RANKL protein expression levels. (C-D) Flow cytometric analysis of cells from murine lung suspensions showed RANKL expression on T cells (C) and epithelial cells (D). More T cells positive for RANKL were found in fibrotic as compared to healthy conditions, while the number of RANKL+ epithelial cells was similar between fibrotic and control conditions. Groups were compared using a Mann-Whitney U test and p<0.05 was considered significant.
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**RANKL is expressed by epithelial cells and T cells in lung tissue**

As OPG is the decoy receptor of RANKL, we assessed RANKL expression patterns in human and mouse lung tissue. We found clear expression of RANKL in both human and mouse lung tissue (Figure 3). Overall RANKL protein levels were similar in fibrotic lung tissue compared with control lung tissue in both humans (Figure 3A) and mice (Figure 3B).

To assess which cells in mouse lung tissue express RANKL, we analyzed RANKL expression in by flow cytometry. We found clear RANKL+ expression on CD3+ T cells and EpCAM+ epithelial cells (Figure 3C and D) and no significant expression on macrophages. Induction of fibrosis by silica resulted in higher number of RANKL+ T cells (Figure 3C) and no change in the number of RANKL+ epithelial cells (Figure 3D).

**RANK expression on lung macrophages in decreased in fibrotic conditions**

RANK, the membrane-bound receptor of RANKL, was also expressed in both human and mouse lung tissue and levels were similar between fibrotic and control lung tissue in both humans and mice (Figures 4A and 4B). When staining for RANK we found expression by bronchial epithelial cells and interstitial as well as alveolar macrophages (Figures 4C-F).

**OPG levels correlated with collagen I levels and are induced by TGF-β**

To assess its relationship with fibrosis, we studied whether OPG levels in murine lung tissue correlated with markers of fibrosis. Indeed, OPG levels in the lung correlated with the collagen-I content of the lung (Figure 5A) and serum OPG levels reflected the OPG levels in the lung (Figure 5B). To confirm OPG is produced by lung tissue under the control of a profibrotic cytokine, we incubated murine precision-cut mouse lung slices with TGFβ, the hallmark cytokine of fibrosis. We found that precision-cut mouse lung slices produced more OPG following TGFβ stimulation than unstimulated slices (Figure 5C). Moreover, 3T3 murine fibroblasts produced detectable levels of OPG at baseline and stimulation with TGFβ resulted in even higher OPG levels in culture supernatant (Figure 5D).
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Figure 4. Western blot analysis of RANK shows expression of this protein in human and murine lung tissue (A-B). No differences were found in RANK expression between control and fibrotic lung tissue in humans (A) and mice (B). Lung tissue lysates were collected from 15 control patients undergoing surgical resection for carcinoma or COPD and 11 samples were collected from pulmonary fibrosis patients undergoing lung transplantation. Mice were either silica-exposed to induce pulmonary fibrosis (n=4) or vehicle-exposed (n=3) for 28 days. (C-D) Immunohistochemical staining for RANK in paraffin-embedded control mouse lung tissue revealed expression by alveolar and interstitial macrophages and bronchial epithelial cells. (E-F) Immunohistochemical staining of fibrotic murine lung tissue showed a similar expression pattern of macrophages and bronchial epithelial cells as for control lung tissue.
Figure 5. (A) OPG levels in lysates of lung tissue from mice exposed to silica correlated with collagen I levels in that same lung tissue. (B) OPG serum levels correlated with OPG levels in murine fibrotic lung tissue. A Spearman correlation coefficient was calculated for each correlation and \( p < 0.05 \) was considered significant. (C) OPG production was higher in murine precision-cut lung slices following 48 hours of TGF-\( \beta \) stimulation compared to vehicle-treated controls (\( n = 7 \) independent experiments). (D) OPG levels in culture supernatant of murine 3T3 fibroblasts were higher after 24 hours of TGF-\( \beta \) stimulation than in supernatant from vehicle-stimulated fibroblasts (\( n = 7 \) independent experiments). Groups were compared using a Mann-Whitney \( U \) test and \( p < 0.05 \) was considered significant.

**RANKL induces ECM-degrading enzymes in macrophages with a hematopoietic origin**

We tested whether RANKL is able to induce a proteolytic phenotype in either macrophages with an embryonic origin (alveolar macrophages) or with a hematopoietic origin (infiltrating monocytes).\(^{20}\) We found that 48 hours of RANKL stimulation induced significant expression of MMP9 mRNA (Figure 6A) and activity of cathepsin K (Figure 6B) in monocyte-derived RAW264.7 macrophages as compared to unstimulated cultures. No polynuclear cells were seen, which would be an indication of osteoclast formation. In contrast, we found that RANKL did not induce MMP9 mRNA expression in the embryonic alveolar-like MPI macrophages (Figure 6C).
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Figure 6. In RAW264.7 monocytic macrophages, MMP9 mRNA (A) and cathepsin K activity (B) were higher following RANKL stimulation (24h and 72h respectively) as compared to unstimulated controls. (C) sRANKL stimulation of MPI alveolar-like macrophages did not result in higher MMP9 expression as compared to unstimulated controls. Groups were compared using a Mann-Whitney U test and p<0.05 was considered significant.

**RANKL treatment of mice with silica-induced pulmonary fibrosis does not reverse fibrosis, but does result in more OPG and epithelial cells**

By treating mice with silica-induced pulmonary fibrosis with recombinant sRANKL, we aimed to overcome the high levels of OPG and induce the development of ECM-degrading macrophages. Induction of fibrosis by silica resulted in higher collagen I deposition and more neutrophils, more macrophages (both interstitial and alveolar) and, as expected, more production of OPG as compared to nonexposed healthy mice (Figures 7A-K). Exposure to silica did not change the number of infiltrating T cells (Figure 7B) or the number of epithelial cells (Figure 7D), but did lead to a higher number of RANKL+ T cells in lung tissue (Figure 7C) as compared to nonexposed healthy mice.
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Figure 7. Mice were treated with sRANKL (3x per week for two weeks, 5 or 10 µg per mouse/administration) four weeks after inducing pulmonary fibrosis with silica. Collagen I deposition (A), numbers of CD3+ T cells (B), RANKL+CD3+ T cells (C), Epcam+ epithelial cells (D), RANKL+Epcam+ epithelial cells (E), neutrophils (F), total macrophages (G), alveolar macrophages (H), interstitial macrophages (I), and levels of OPD mRNA expression (J) and protein expression (K) were assessed. Silica-exposure led to higher collagen I deposition in lung tissue, more RANKL+CD3+ T cells, neutrophils, total, interstitial and alveolar macrophages, and higher expression of OPG mRNA and protein in lung tissue as compared to nonexposed animals. sRANKL treatment did not change collagen I deposition but did dose-dependently increase the number of (RANKL+) epithelial cells and also resulted in higher OPG mRNA and protein levels in lung tissue as compared to mice with untreated fibrosis. Groups were compared using a Kruskall Wallis test for multiple testing and p<0.05 was considered significant. (L) OPG mRNA expression did not change in TGFβ + sRANKL-treated murine precision-cut lung slices compared to TGFβ + vehicle-treated controls (n=6 independent experiments).

Treatment with 5 or 10 µg sRANKL had no effect on collagen I deposition and the number of (RANKL+) T cells, neutrophils, and (alveolar and interstitial) macrophages as compared to silica-exposed nontreated animals. Collagen Iα1, MMP9 and fibronectin mRNA expressions also increased after silica exposure and remained unchanged following sRANKL treatment (Supplemental Figures 2A, 2B and 2C).

sRANKL treatment did result in a strong induction of OPG expression (Figure 7J) and this also resulted in higher OPG levels in lung tissue as compared to silica-exposed nontreated animals (Figure 7K). In addition, sRANKL treatment resulted in a dose-dependent significant increase in the number of epithelial cells in lung tissue as compared to silica-exposed nontreated animals and this was paralleled by a similar pattern for the number of RANKL+ epithelial cells (Figures 7D and E).

To investigate whether sRANKL could directly induce the expression of OPG in lung tissue, we incubated precision-cut murine lung slices with TGFβ for 72 hours and added sRANKL during the last 24 hours to mimic our in vivo experiment. sRANKL treatment for 24 hours did not lead to higher expression of OPG mRNA in lung slices as compared to nontreated slices (Figure 7L).
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DISCUSSION

Our study shows that OPG protein levels are higher in lung tissue of both humans and mice with (silica-induced) pulmonary fibrosis and that in addition to OPG, its ligand RANKL and the membrane-bound receptor RANK are all present in lung tissue. In mice with silica-induced fibrosis, these lung tissue levels of OPG correlate positively with the amount of collagen in the lung. The findings that sRANKL treatment increases epithelial cell numbers and OPG expression in lung tissue suggests that the RANK/RANKL/OPG axis is not only active in bone, but also has a role in regulating tissue repair processes in the lung.

OPG has previously been found to associate with fibrosis-related diseases like vascular fibrosis21, cystic fibrosis22 and liver fibrosis11,12. In addition, high OPG levels were found bronchoalveolar lavage fluid of mice with silica- and bleomycin-induced fibrosis15,16 and in a model of endotoxin-induced airway remodeling15. We now show that OPG expression is also high in human fibrotic lung tissue and that it is produced by fibroblasts isolated from this lung tissue. The higher OPG production resulted from production in lung tissue itself, as OPG mRNA expression in lung tissue was also higher in experimental fibrosis. Some have suggested that OPG levels are higher to counteract increased RANKL levels that may be elevated due to the actions of IL-6 and TNF-α23. However, we showed that TGFβ, the hallmark cytokine of fibrosis, is capable of increasing OPG production. Therefore, our findings strongly support the concept that OPG is associated with the fibrotic process itself and is produced locally in the lung.

In bone, OPG prevents the development of osteoclasts by binding RANKL to reduce bone ECM degradation. We hypothesized a similar role for OPG in the lungs. However, when treating with sRANKL to overcome the excess OPG in fibrosis, we found no differences in collagen I deposition in lung tissue, our outcome measure for pulmonary fibrosis. In contrast, sRANKL treatment even further induced OPG levels, potentially still preventing the induction of ECM degradation. Possibly, the sRANKL administered binds to free OPG, triggering a pulmonary or extrapulmonary feedback mechanism to compensate for the lower levels of free OPG. To explain this phenomenon, we studied whether RANKL could directly induce OPG in precision-cut lung slices and found it did not change OPG mRNA expression. It therefore seems likely that RANKL somehow indirectly induces the expression of OPG in lung tissue. Clearly,
The RANK/RANKL/OPG axis has a role in regulating tissue repair processes in lung.

Our findings have provided interesting leads to further investigate the regulation of OPG in the lung. Interestingly, the treatment with sRANKL clearly was able to affect cells within lung tissue as we found a strong dose-dependent increase in the number of Epcam+ cells, which is indicative of an increase in the number of epithelial cells or an upregulation of the Epcam protein by these cells. Immunohistochemical staining for RANK showed expression of this receptor of RANKL on bronchial epithelial cells and these cells could therefore potentially be responsive to the effects of RANKL. To the best of our knowledge no reports on RANK expression on lung epithelial cells have been published nor on the effects of stimulating this receptor with RANKL in lung tissue. However, both mammary epithelial cells as well as a subset of thymic epithelial cells were found to expand after exposure to RANKL\textsuperscript{24,25} which may suggest RANKL is a more general epithelial growth factor. Further studies investigating proliferation effects of RANKL on bronchial and alveolar epithelial cells are needed to address this question. In combination with the clear pulmonary regulation of OPG during fibrosis, these data do suggest a RANK/RANKL/OPG axis is operating in lung tissue during tissue repair.

RANKL could theoretically still play a role in the resolution of fibrosis by stimulating the expression of ECM-degrading enzymes in macrophages. However, its usefulness seems limited because of its ability to upregulate OPG production. In vitro stimulation of embryonic alveolar-like macrophages with sRANKL did not induce MMP9 expression, but it did induce a proteolytic phenotype in monocyte-derived RAW264.7 macrophages as shown by our data and those of others previously\textsuperscript{20,26-32}. MMP9 and cathepsin K can both contribute to the ECM degradation process\textsuperscript{6,7,33} and these monocyte-derived macrophages may be therapeutically more relevant as Gibbons et al. previously showed that infiltrating monocyte-derived macrophages contributed largely to the development of fibrosis, whereas resident alveolar macrophages did not\textsuperscript{9}. The development of ligands that induce this proteolytic state in monocyte-derived macrophages without inducing OPG production may be a novel way of inducing ECM degradation and treating advanced fibrotic disease\textsuperscript{34}.

Based on our findings that serum OPG levels correlated significantly with OPG levels in murine lung tissue, OPG may be considered as a biomarker of disease severity.
or treatment effects. OPG serum levels in patients with liver cirrhosis have been included into a panel of serum biomarkers to improved diagnostic accuracy of a noninvasive test to assess liver fibrosis severity\textsuperscript{35,36}. OPG may be similarly useful in pulmonary fibrosis based on our \textit{in vivo} data, even though McGrath \textit{et al.} found no differences between controls and IPF patients OPG serum levels and no association with lung function parameters\textsuperscript{37}. Further studies could aim at investigating whether OPG correlates with non-invasive diagnostic parameters for (early) detection of the disease or with disease progression.

A limitation of our findings is the fact that our mouse model of silica-induced pulmonary fibrosis only represents one cause of pulmonary fibrosis; while in humans the causes are diverse and usually not known. The patients from whom the lung tissue samples were obtained, however, represented many types of pulmonary fibrosis (idiopathic pulmonary fibrosis, fibrotic non-specific interstitial pneumonia and progressed chronic extrinsic allergic alveolitis). All samples showed high levels of OPG, while we found low levels of OPG in COPD and in control lung tissue obtained from patients undergoing surgical resection for carcinoma with normal lung function. This suggests that the induction of OPG production is specific for fibrotic lung diseases, though other lung diseases should be considered to confirm this assumption.

RANKL is just one of the ligands for OPG and we focused on in relation to fibrosis since RANKL possesses the highest affinity for OPG out of all other possible ligands\textsuperscript{38}. OPG is expressed in various organs and can also function as a decoy receptor of TNF-related apoptosis-inducing ligand (TRAIL), heparin and glycosaminoglycans\textsuperscript{35,36}. Involvement of one of these ligands may also explain possible actions of OPG in the lung or generally in fibrosis\textsuperscript{39,40}. For example, by binding TRAIL, OPG may be able to prevent TRAIL-induced apoptosis of lung myofibroblasts and thereby allowing myofibroblasts to continue producing ECM\textsuperscript{37,41}. To investigate all ligands together instead of investigating them separately, a lung-specific conditional OPG knockout mouse could be considered in combination with models of experimental pulmonary fibrosis to determine the impact of OPG on the development and onset of fibrosis.

In conclusion, OPG expression is higher in fibrotic lung tissue as compared to control lung tissue and the profibrotic mediator TGF\(\beta\) induces its production by
The RANK/RANKL/OPG axis has a role in regulating tissue repair processes in lung fibroblasts. The induction of OPG and the increase in epithelial cell numbers in lung tissue following sRANKL treatment of pulmonary fibrosis suggest that the RANK/RANKL/OPG axis is not only active in bone, but also has a role in regulating tissue repair processes in the lung. Even though the exact role of OPG in fibrosis remains undetermined, the fact that OPG production is closely linked to fibrosis makes it an interesting new avenue to explore for drug and biomarker development.

ACKNOWLEDGEMENTS

The Pender Foundation is gratefully acknowledged for their support of the exchange of fibrotic lung tissue between the Groningen and Rotterdam centers (BM and BB). A Young Investigator Award of the Netherlands Respiratory Society for our research on OPG in pulmonary fibrosis is gratefully acknowledged (CB). Barbro N Melgert is an active member of COST action BM1201. We would like to thank the following Bachelor students of Pharmacy and Master students of Pharmacy for their practical contributions to the manuscript: Burak Güney, Axel Haak, Annemarie Broesder, Thirsa Tuin and Marcelina Stel.
REFERENCES


The RANK/RANKL/OPG axis has a role in regulating tissue repair processes in lung


SUPPLEMENTARY DATA

MATERIALS AND METHODS

Human tissue
Human fibrotic lung tissue was collected with informed consent from patients with end-stage pulmonary fibrosis undergoing lung transplantation at either the University Medical Center Groningen (UMCG) or at the Erasmus Medical Center Rotterdam. In Groningen, the study protocol was consistent with the Research Code of the University Medical Center Groningen (http://www.umcg.nl/EN/Research/Researchers/General/ResearchCode/Paginas/default.aspx (accessed 18 Feb 2016)), and Dutch national ethical and professional guidelines (http://www.federa.org (accessed 18 Feb 2016)). In Rotterdam, the Medical Ethical Committee approved all protocols followed in that center.

Control lung tissue was obtained at the UMCG from patients undergoing surgical resection for carcinoma or chronic obstructive pulmonary disease (COPD). In the cases of tumor resections, histologically normal lung tissue was taken as far distally as possible from the tumor and assessed visually for abnormalities with standard haematoxylin and eosin staining.

Isolation and culture of primary human fibroblasts
Lung fibroblasts were isolated from lung explants using a technique described previously [18, 19]. In short, fresh parenchymal lung tissue, excluding visible vessels and airways, was cut into 1-2 mm pieces that were then cultured for 4-5 weeks in 12-well plates in the presence of complete Ham’s F12 medium [10% fetal calf serum (FCS, Invitrogen, The Netherlands), Penicillin/Streptavidin, fungizone, glutamine] at 37°C in an atmosphere of 5% CO2. At 25% confluency, the cells were transferred to T25 flasks. After 1 week, the cells were plated in 12-wells plates (50,000 cells/well) for experiments or cryopreserved in FCS with 10% DMSO under slow cooling conditions and eventually stored at -150°C. Cells used in the experiments described were maximally passaged three times. Plated cells were grown to confluence and transferred to low-serum medium (0.5% FCS). After 72h, supernatants were collected for ELISA analysis of OPG levels and stored at -80°C for later analysis.

Animal experiments
Male C57BL/6 mice were obtained from Harlan (Zeist, The Netherlands). Animals were maintained with permanent access to food and water in a temperature-controlled environment with a 12h dark/light cycle regimen. All animal experiments were approved by the Institutional Animal Care and Use Committee. Mice were used in experiments with silica-induced pulmonary fibrosis (DEC6064) and for the preparation of precision cut lung slices (DEC6416AA). Animal experiments were performed in the animal facility of the University of Groningen according to strict governmental and international guidelines on animal experimentation.

Silica-induced lung fibrosis in mice
Fibrosis was induced using Min-U-Sil 5 crystalline silica (a kind gift from Dr. Andy Ghio, US EPA, Chapel Hill, NC). Animals were anesthetized using isoflurane before they received a single administration of crystalline silica (0.2 g/kg) in 50 μl 0.9% saline by intratracheal
 installation, while control animals received an equivalent volume of 0.9% saline. Mice were sacrificed after 28 days and serum and lung tissue were collected (n=6-8).

In mice treated with sRANKL, silica was administrated on day zero and sRANKL treatment began after 28 days and was continued for an additional 2 weeks. We calculated that the administered sRANKL dose should exceed the calculated OPG content (0.8 μg OPG/lung) in fibrotic lungs to be able to see a biological effect of sRANKL. We therefore used doses of 5 μg (n=12) and 10 μg RANKL (n=12) per mouse three times per week. sRANKL was administered intranasally in 40 μl saline. Control mice with pulmonary fibrosis received 40 μl saline (vehicle) three times per week (n=12). On day 43, mice were sacrificed and lungs were collected for flow cytometry, western blot analysis, ELISA, or histology. See Figure 1 for an overview of the experimental setup.

Mouse lung precision-cut lung slices

An ex vivo model of early lung fibrosis development was used to investigate the effect of TGFβ stimulation on OPG production by lung tissue. Lungs of male C57BL/6 mice (20-30gr, age 6-8 weeks) of in total six mice were used to make precision-cut lung slices. After sacrifice by exsanguination via the aorta abdominalis under isoflurane anaesthesia, mouse lungs were filled with 1.5% low-melting temperature agarose in 0.9% NaCl (Sigma-Aldrich) and transferred directly into ice-cold University of Wisconsin organ preservation solution (DuPont Critical Care, Waukegab, IL). Lung slices of 5-mm in diameter and 10-15 cell layers thickness, were prepared with a Krumdieck tissue slicer (Alabama Research and Development, AL) using ice-cold Krebs-Henseleit Buffer [25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO3 (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH), saturated with carbogen (95% O2/5% CO2) and adjusted to pH 7.4] as we have previously described20.

Lung slices were incubated in 12-well plates in DMEM + Glutamax medium [4.5g/L D-glucose and pyruvate (Gibco) supplemented with a non-essential amino acid mixture (1:100), 100U/ml penicillin, 100µg/ml streptomycin, 45 µg/ml gentamycin and 10% FCS]. After a 1h pre-incubation at 37°C in a 95% O2/5% CO2 atmosphere with continuous shaking at 90 rpm, the slices were transferred into fresh medium and incubated in triplicate for 48h with TGFβ (5 ng/ml) or 48h with TGFβ followed by either vehicle or RANKL (200ng/ml) stimulation for 24 hours. Three slices from each condition were pooled for further analyses and tissue slice supernatants were collected separately. All samples were stored at -80°C for further analyses.

Mouse cell cultures

NIH 3T3 fibroblasts (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, The Netherlands) [10% FCS, penicillin/streptomycin] at 37°C in an atmosphere of 5% CO2.

RAW 264.7 macrophages (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, The Netherlands) [10% FCS, L-Glutamine, Gentamycin] and self-propagating mouse alveolar-like macrophages (MPI cells, a kind gift from Dr. G. Fejer, Plymouth University, Devon, UK) were cultured in RPMI 1640 medium (Gibco, Bleiswijk, The Netherlands) [10% FCS, 20 ng/ml GM-CSF]. Macrophages were cultured at 37°C in an atmosphere of 5% CO2.
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3T3 cells were stimulated with TGFβ to transform them to myofibroblasts. Before TGFβ stimulation, plated cells were incubated in 0.5% FCS medium. After 24h, cells were stimulated with TGFβ (5 ng/ml, Peprotech, Rocky Hill, NJ) for 48h. Afterwards, the supernatant was collected and stored at -80°C until further use.

To induce a proteolytic phenotype, RAW macrophages (until max. passage 12) or MPI macrophages were stimulated with 200 ng/ml soluble RANKL (sRANKL, produced at our own facility. Technical details are described in “Production and purification of sRANKL protein”). Cells were lysed after 24h for the isolation of mRNA or after 72 hours for protein analysis. Samples were stored at -80°C for later analysis. For each experiment, the data presented are representative of at least three independent replicates.

**Protein isolation from lung tissue**

Protein was isolated from frozen human or mouse lung tissue (30-40 mg) in buffer (0.25 M Tris/HCl buffer, 2.5% Igepal, 0.5% SDS, Protease Inhibitor Cocktail (Boehringer Mannheim, Germany), pH 7.5) by incubating for 1 hour at 4°C, homogenizing and centrifuging at 13,200 rpm for 30 min. Supernatants were collected and the overall protein concentration of the tissue lysates was determined using an RC DC™ Protein Assay based on the Lowry method (cat.#5000111, Bio-Rad, Hercules, CA). Samples were stored at -80°C until further analyses by Western blot or ELISA analysis.

**Western blot analysis**

Western blot analysis was used to compare levels of mouse and human RANK and RANKL in control and fibrotic lung tissue. One hundred micrograms of total protein per sample was loaded into the well of a 10% SDS-PAGE gel and transferred to a polyvinylidene-fluoride membrane. Membranes were blocked with 5% nonfat milk in Tris-Buffered saline with 0.05% Tween 20 (TBST) for 2 hours, following incubation with the first antibody in blocking buffer overnight at 4°C. The following primary antibodies were used: rabbit-anti-mouse RANK (1:200, Acris, Herford, Germany), goat-anti-human RANK (1:100, R&D Systems, Minneapolis, MN), rabbit-anti-RANKL (human and mouse, 1:100, Acris) and mouse-anti β-actin (1:20,000 Sigma-Aldrich) as a loading control. After washing 30 min with TBST, the membrane was incubated with an appropriate secondary antibody in blocking buffer for 2 hours at room temperature. Subsequently, membranes were washed with TBST and with TBS before the bands were visualized with enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA). Band intensity was measured and quantified using GeneSnap (SynGene, Synoptics, Cambridge, UK). RANK and RANKL expression levels were normalized to β-actin expression.

**ELISA**

Human and mouse OPG levels in lung tissue and culture supernatants were assessed using ELISA (cat#DY805 (human), cat#DY459 (mouse), R&D Systems) according to the instructions provided by the manufacturer. One hundred micrograms of tissue lysate total protein in a total of 100 μl was analyzed per sample. Cell culture media was analyzed undiluted while lung slice culture medium was diluted 1:5 before analysis.
Immunohistochemistry

Immunohistochemical analysis of collagen I, RANK, and OPG was performed on 3 µm paraffin sections of mouse lung tissue. Tissue sections were deparaffinized in xylene, rehydrated in alcohol, rinsed in milliQ water and placed in PBS. When applicable, sections were subjected to antigen retrieval (0.1 N Tris-HCl pH 9.0 buffer, overnight at 80°C). Prior to the incubation with the OPG antibody, sections were blocked with 1% BSA in 5% nonfat milk (Sigma-Aldrich). Primary antibodies were then incubated for 1h at room temperature in the presence of 5% normal mouse serum. Antibodies used were goat-anti-mouse collagen I (1:75, Southern Biotech, Birmingham, AL), rabbit-anti-mouse OPG (1:400, Antibodies-online, Atlanta, GA), and goat-anti-human RANK (1:100, R&D Systems). Primary antibody incubation was followed by incubation with species-specific PO-labeled secondary and optional tertiary polyclonal antisera. PO-labeled antibodies were visualized using ImmPACT NovaRED kit (Vector, Burlingame, USA) with a hematoxylin counterstain when appropriate.

The amount of collagen deposition in the lung was determined using ImageScope software (Aperio, Burlingame, USA). After selecting the stained area of the lung sections excluding edges of the tissue, large airways and vessels, a threshold was set to identify positive staining of the tissue (represented by collagen). The percentage of stained-tissue surface per total-tissue surface analyzed was then calculated for each section.

Cathepsin K activity

Cathepsin K activity of RAW264.7 macrophages was measured using a Cathepsin K Activity Kit (Cat#KA0769, Abnova, Taipei, Taiwan) following instructions provided by the manufacturer. Cathepsin K activity was normalized against the activity observed in unstimulated cells.

Quantitative Real-time PCR

Total mRNA was isolated from cells or tissues using a Maxwell® LEV simply RNA Cells/Tissue kit (cat# AS1280, Promega, Madison, WI) according to the instructions provided by the manufacturer. Final mRNA concentrations were determined using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE). All primers were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), as described in Supplemental Table 1. Transcription levels of these genes were measured by using 20 ng cDNA per sample in a quantitative real-time PCR (SensiMix™ SYBR kit (Bioline, Taunton, MA)) and an ABI7900HT sequence detection system (Applied Biosystems, Foster City, CA). For each sample, the threshold cycles (Ct values) were calculated using SDS 2.3 software (Applied Biosystems), and mRNA expression was normalized to β-actin for cell culture mRNA samples, YWHAZ for tissue mRNA samples, and 18S for precision-cut murine lung slices. Results are expressed as fold-change of healthy controls or control conditions.

Production and purification of sRANKL protein

Plasmid pET15b-mRANKL, encoding the extracellular domain of murine RANKL (sRANKL; amino acids 160-316), was transformed into Escherichia coli strain BL21(DE3). A 10 ml overnight culture was used to inoculate 1L 2xYT [16g Bacto Tryptone, 10g Bacto Yeast Extract, 5g NaCl, pH 7.0] medium containing 0.1mg/l ampicillin. Cells were grown at 37°C until
the absorbance at 600 nm was 0.5 after which sRANKL production was induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside and incubation continued for another 16 hours at 20 °C.

After harvesting and resuspending wet cells at 1 g/3 ml in buffer A [50 mM MES, pH 5.8, 2 mM dithiothreitol, 10 % glycerol], cells were carefully sonicated on ice. Cell debris was removed by centrifugation for 60 min at 38,000xg and 4 °C. Supernatants were loaded on an 8 ml cation exchange column (Source 30S, GE Healthcare, USA). sRANKL eluted early from a linear 0-500 mM NaCl gradient in buffer A. Pooled fractions at 4 °C were brought to pH 7.5 by adding drops of 1 M NaOH and to 1.3 M (NH₄)₂SO₄ by slowly adding pulverised salt crystals. The protein was loaded on a 5 ml HiTrap phenyl sepharose column (GE Healthcare), pre-equilibrated with buffer B [20 mM sodium phosphate buffer, 2 mM dithiothreitol, 10 % glycerol, pH 7.5] and eluted early from a linear 1.3-0 M (NH₄)₂SO₄ gradient in buffer B. Pooled fractions were run on a HiTrap Superdex75 column (GE Healthcare) in 20 mM sodium phosphate buffer NaPi, [NaH₂PO₄, Na₂HPO₄, 10 % glycerol, pH 7.5]. Protein purity was checked by SDS-PAGE and sRANKL concentrations were measured with a Coomassie Bradford protein assay kit (Pierce, USA) using BSA as reference and with a RANKL ELISA kit (cat#DY462, R&D Systems). Values from both assays were always in the same range and the concentration obtained from the ELISA analysis was used for further experiments. Purified RANKL is routinely tested for endotoxin contamination comparing heat-inactivated RANKL with native RANKL using RAW264.7 macrophages and no measurable contamination was found.

**Flow cytometry**

Mouse left lungs were minced and incubated in RPMI containing 10% FCS, 10 μg/ml DNase I (grade II from bovine pancreas, Roche Applied Science, Almere, The Netherlands), and 0.7 mg/ml collagenase A (Sigma-Aldrich) in a shaking water bath (37°C) for 45 min. Digested lung tissue was mashed through a 70 μm nylon strainer (BD Biosciences, Breda, The Netherlands) resulting in a single cell suspension. To remove contaminating erythrocytes, the cells were incubated with Pharmlyse (BD Biosciences) for 2 min at room temperature. Total cell numbers were determined using a FACS Array cell counter (BD Biosciences) and used to calculate total numbers of specific cell types.

Single lung cell suspensions were stained for T cells, neutrophils, epithelial cells and macrophage subsets by flow cytometry (see Supplemental Table 2 and 3 for antibody details). A viability dye was included (Fixable Viability Dye eFluor® 506, eBioscience, San Diego, CA) to exclude dead cells. T cells were identified using anti-CD3-APC/Cy7 and epithelial cells were identified using anti-EpCAM-Alexafluor 647. Interstitial macrophages were identified as CD68+CD64negCD11c-low-expressing cells and alveolar macrophages were identified as CD68+CD64varCD11C-high-expressing cells using anti-CD68-PerCp/Cy5.5, anti-CD64-PE/Cy7 and anti-CD11-Brilliant Violet 785. Neutrophils were identified as CD68negGR1+ cells. In addition, the expression of RANKL on T cells, epithelial cells, and macrophages was determined by using anti-RANKL-FITC. See supplemental data Figure 3 and 4 for the gating strategy.

Approximately 10⁶ cells were incubated with cell surface marker antibodies in the presence of 1% normal mouse serum (30 min, on ice, protected from light). The cells were then washed with PBS containing 2% FCS and 5 mM EDTA and fixed and permeabilized using a
The RANK/RANKL/OPG axis has a role in regulating tissue repair processes in lung

fixation and permeabilization buffer (30 min, eBioscience) to stain for intracellular markers. After washing the cells with permeabilization buffer, all samples were resuspended in FACS lysing solution (Biosciences) and stored at 4 °C until analysis. Samples were analyzed using an LSRII flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (Tree Start, Ashland, USA). For each antibody, a Fluorescent Minus One (FMO) control was included for proper gating during data analyses.

Statistical analysis

All groups were considered non-normally distributed due to small samples sizes. Therefore, statistical differences between two groups were assessed using nonparametric Mann-Whitney U tests. For the comparison of multiple groups, we used Kruskal Wallis. When significant, a Mann-Whitney-U test with a Bonferroni adjustment for multiple comparisons was used as a post-test to calculate statistical differences between specific groups. Correlations between OPG levels in lung and serum, and the correlation with collagen content in the lungs were assessed by calculating a nonparametric Spearman correlation coefficient (R). Significance was considered *= p<0.05. The data were analyzed using GraphPad Prism 6.
Supplemental Table 1. Sequences of primers

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Supplemental Table 2. Overview of flow cytometry antibodies used to stain for T cells and epithelial cells.

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Supplemental Table 3. Overview of flow cytometry antibodies used to stain for macrophages.

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Supplemental Figure 1. A. Collagen I mRNA expression was higher in lungs of mice exposed to silica (fibrosis, n=7) as compared to healthy controls (n=8). Levels were corrected for YHWAZ as housekeeping gene. B. Representative pictures of an immunohistochemical staining for collagen I deposition, showing that collagen I deposition was higher in the lungs of mice exposed to silica as compared to saline-exposed controls. Collagen I expression is indicated by the red colour.
**Supplemental Figure 2.** mRNA expressions of collagen Iα1 (A), fibronectin (B) and MMP9 (C) were higher in fibrotic mouse lungs following silica exposure for 28 days (n=6) and vehicle treatment for two weeks as compared to healthy controls (n=3), but unchanged in fibrotic lungs of mice that received sRANKL treatment for two weeks (5μg/mouse, n=6 and 10μg/mouse, n=6) as compared to vehicle treatment. Groups were compared using a Kruskall Wallis test for multiple testing and p<0.05 was considered significant.
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**Supplemental Figure 3.** Gating strategy for T cells and epithelial cells.

**Supplemental Figure 4.** Gating strategy for macrophages and neutrophils.
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