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ON THE CROSSROADS OF INNATE IMMUNITY AND ARTHRITIS

Impact of Interleukin-1, Fc gamma receptors and LDL-cholesterol on myeloid cells and joint pathology

Giuliana Ascone
The research presented in this thesis was carried out at the Department of Experimental Rheumatology, Radboud university medical center (Radboudumc), within the Radboud Institute for Molecular Life Sciences (RIMLS), Nijmegen- the Netherlands, and in close collaboration with Future Diagnostics solutions (FDx), Wijchen- the Netherlands. This work was supported by a grant from Marie Curie Initial Training Network (Euroclast- FP7-People-2013-ITN: No 607447).

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ON THE CROSSROADS OF
INNATE IMMUNITY AND ARTHRITIS

Impact of Interleukin-1,
Fc gamma receptors and
LDL-cholesterol on myeloid cells
and joint pathology

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# Table of contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td>General introduction and outline of the thesis</td>
<td>12</td>
</tr>
<tr>
<td><strong>Chapter 2</strong></td>
<td>Enhanced IL-1 signaling <em>in vivo</em> steers the number of monocyteic osteoclast precursors, osteoclastogenesis and resorptive activity dependent on the skeletal location: implications for RA and PD pathology</td>
<td>29</td>
</tr>
<tr>
<td><strong>Chapter 3</strong></td>
<td>Fcγ receptor-mediated influx of S100A8/A9-producing neutrophils as inducer of bone erosion during antigen-induced arthritis</td>
<td>49</td>
</tr>
<tr>
<td><strong>Chapter 4</strong></td>
<td>High LDL-C levels attenuate onset of inflammation and cartilage destruction in antigen-induced arthritis</td>
<td>79</td>
</tr>
<tr>
<td><strong>Chapter 5</strong></td>
<td>High LDL levels lessen bone destruction in antigen-induced arthritis by inhibiting osteoclast formation and function</td>
<td>101</td>
</tr>
<tr>
<td><strong>Chapter 6</strong></td>
<td>Human APOE-ε4 results in more severe experimental osteoarthritis in comparison to APOE-ε3; APOE-isoforms as risk factor for osteoarthritis development</td>
<td>125</td>
</tr>
<tr>
<td><strong>Chapter 7</strong></td>
<td>Genetic modification of ER-Hoxb8 osteoclast precursors using CRISPR/Cas9 as a novel way to allow studies on osteoclast biology</td>
<td>135</td>
</tr>
<tr>
<td><strong>Chapter 8</strong></td>
<td>Summary and final considerations</td>
<td>155</td>
</tr>
<tr>
<td><strong>Chapter 9</strong></td>
<td>Samenvatting</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>Sintesi</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>List of Publications</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>Acknowledgements</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>RIMLS Portfolio</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Curriculum Vitae</td>
<td>193</td>
</tr>
</tbody>
</table>
CHAPTER 1

General introduction
CHAPTER 1

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disease of the joints that affects 0.5-1% of the population world-wide. Persistent systemic and local inflammation in the joints causes chronic activation of the immune system, leading to progressive damage of cartilage and bone, and ultimately to joint dysfunction. Although many factors are known to contribute to the etiopathogenesis of the disease, the exact cause remains not fully understood. Whereas there is still no actual cure for RA, great progress has been made in developing effective therapies, predominantly focusing on preventing or limiting joint damage, reducing pain, and optimizing life quality. Therefore, further research is needed to identify new mechanisms and factors involved in the pathogenesis of the disease. This could lead to the discovery of new therapeutic targets to interfere with the development of auto-immunity and the establishment of a vicious circle, allowing to treat earlier stages of the disease and significantly improve patients’ outcome.

As RA is an autoimmune disease, the activation of the cells of the immune system plays a central role in its pathogenesis. B cells, T cells, neutrophils and macrophages tightly regulate the pathological processes observed in RA, characterized by chronic inflammation of the joints, pannus formation, and consequent cartilage and bone destruction. Auto-reactive B cells present in the blood stream and in the synovium lead to the production of autoantibodies forming immune-complexes (ICs), mainly consisting of immunoglobulin G (IgG) antibodies with their cognate antigen. The presence of such ICs is clearly associated with the initiation and progression of RA. In particular, the binding of ICs to Fcγ receptors (FcγRs) present on neutrophils and macrophages strongly stimulates the innate immune response, which activation is essential for the onset and perpetuation of the inflammatory response. Besides, B cells serve as strong stimulators of T cell activity and together trigger various effector functions of innate immune cells (Figure 1), including the massive production of cytokines and chemokines that drive joint inflammation and bone destruction.

Osteoclasts and bone destruction

Bone erosion is mediated by osteoclasts, multinucleated cells differentiated from myeloid precursors of the monocyte-macrophage lineage under the influence of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB (RANK) ligand (RANKL). The activation of the RANKL/RANK signaling axis induces the expression of nuclear factor of activated T cell 1 (NFATc1), a transcriptional factor crucial to increase the intracellular levels of calcium (Ca²⁺) needed during osteoclastogenesis. This results in the further differentiation of precursor cells and their fusion into multinucleated osteoclasts. The latter are characterized by expression of a
series of osteoclast-specific markers such as tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, chloride channel-7, matrix metalloproteinase 9 (MMP-9), carbonic anhydrase II, and cathepsin K. During the differentiation process the cells become polarized and undergo rearrangements of the actin cytoskeleton forming tight junctions known as ‘actin rings’ to form a sealed zone. This compartment is rapidly acidified due to the generation of hydrogen ions and the secretion of lytic enzymes that are crucial to resorb bone. Finally, osteoclast activity is induced by the formation of the ruffled border, a convoluted membrane within the sealing zone, that is essential for the resorption of the underlying bone.

Under physiological conditions, bone resorption is always balanced out by bone formation and represent a tightly regulated process. The inflammatory milieu present during RA disrupts the balance between resorption and formation. Auto-reactive B and T cells, as well as activated synovial fibroblasts, are important sources of RANKL, causing enhanced osteoclast formation and bone destruction at the affected joints. RANKL/RANK signaling quickly stimulates bone resorption in vivo by activating pre-existing osteoclasts. Therefore, RANK inhibitors (e.g.: Denosumab) have often been used to reduce bone pathologies characterized by excessive bone destruction. In addition, in the arthritic joint the presence of a large amount of pro-inflammatory mediators like interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), IL-17, and S100A8/A9 strongly fosters inflammation, thus enhancing RANKL-mediated bone destruction. IL-1 increases the survival rate of mature osteoclasts both in vitro and in vivo. Furthermore, several studies showed that TNF-α at least partially drives osteoclastogenesis in RA by acting on the stromal environment to enhance expression of RANK and other osteoclastogenic factors. Moreover, TNF-α amplifies inflammation-driven cartilage and bone destruction.

Figure 1. Schematic representation of a healthy joint (left) and pathological changes (right) in an inflamed arthritic joint. The latter is hallmarked by synovial thickening and consequent increased formation of blood vessels that allows a massive influx of inflammatory cells that produce pro-inflammatory cytokines and chemokines, thereby promoting inflammation-driven cartilage and bone destruction.
the effects of RANKL via activation of nuclear factor kappa-light-chain-enhancer of
differentiated B cells (NF-κB) signaling, which in turn induces NFATc1 \(^{30}\) that is needed to
promote osteoclast differentiation and proliferation \(^{31}\).

Next to the RANKL/RANK signaling, the immunoreceptor tyrosine-based activating motif
(ITAM)-signaling provides co-stimulatory signals that increase the intracellular levels of
\(\text{Ca}^{2+}\) required to induce NFATc1 \(^{32}\). Various immunoreceptors, such as osteoclast-
associated receptor (OSCAR), paired immunoglobulin-like receptor A (PIR-A), and
triggering receptor expressed on myeloid cells 2 (TREM-2) expressed on osteoclasts and
their precursors play a key role in the co-stimulation pathway. Their binding to ITAM
motifs present in the \(\gamma\)-chain and DNAX adaptor protein of 12 kDa (DAP-12) has been
shown to modulate osteoclastogenesis both \textit{in vitro} and \textit{in vivo} \(^{33-35}\). In contrast, the
presence of immunoreceptor tyrosine-based inhibitory motif (ITIM) domains in the
paired immunoglobulin-like receptor B (PIR-B) provides inhibitory signals that
counteract the osteoclastogenic effect of the ITAM-signaling \(^{36, 37}\). DAP12 deficient
\((\text{Tybobp}^{-/-})\) mice show increased bone mass (osteopetrosis) and impaired
osteoclastogenesis. Mice deficient for both \(\text{Tybobp}^{-/-}\) and \(\gamma\)-chain \((\text{Il2r}^{-/-})\) have severe
osteopetrosis and bone marrow (BM)-derived osteoclast precursors (OCPs) from these
mice are unable to differentiate into mature osteoclasts independently of RANKL and
M-CSF stimulation \(^{36, 38}\). Furthermore, OSCAR signals via the \(\gamma\)-chain to increase the levels
of \(\text{Ca}^{2+}\), thereby further promoting osteoclast formation and survival \(^{32, 39}\). Moreover the
use of recombinant soluble OSCAR, which inhibits the receptor function, has also been
shown to inhibit osteoclast differentiation from peripheral blood mononuclear cells
(PBMCs) in the presence of RANKL, M-CSF, and transforming growth factor beta (TGF-\(\beta\))
\(^{40}\). Together these findings strongly suggest an important role of ITAM-co-stimulation
in osteoclastogenesis under physiologic conditions. Interestingly, in active RA patients
higher levels of TREM2, DAP12, OSCAR, and \(\gamma\)-chain were found compared to synovial
tissues from inactive RA, osteoarthritis (OA), or control healthy joints \(^{31}\). Moreover, the
expression of DAP12 in peripheral blood monocyte cells (PBMCs) of active RA patients
was significantly higher in active RA patients than in inactive RA \(^{41}\), suggesting an
important role of the ITAM-costimulation pathway in regulating bone destruction during
inflammatory arthritis.
Immune-complexes and Fcγ receptors in rheumatoid arthritis

As previously described, a large amount of autoantibodies, particularly of the IgG isotype, is often found both in the serum and synovial fluid of RA patients. This leads to the formation of ICs that are crucial to driving the autoimmune response. Anti-citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF) are the most prominent autoantibodies forming ICs and their presence significantly correlates with disease activity and severity of joint destruction \(^2\), \(^42\)-\(^44\). Thus, the interaction of these IgGs with their receptors, the FcγRs, present on cells of the innate immune system, including monocytes, macrophages, neutrophils, and osteoclasts is crucial to the onset and progression of RA \(^45\)-\(^48\).

FcγRs as hub linking innate and adaptive immunity

In humans there are eight different FcγRs, the activating FcγRIA/ IB/ IC, FcγRIIA/ IIC, and FcγRIIIA/ IIIIB, and the inhibiting FcγRIIB, whereas in mice there are four FcγRs (activating FcγRI, FcγRIII, FcγRIV, and inhibiting FcγRIIb) \(^31\). Despite the genes encoding for these receptors do not completely overlap, similarly to humans, in mice the activating FcγRs induce cell activation upon binding to ICs, while the inhibiting FcγRIIb inhibits cell activation, thereby tightly regulating cell function. Thus, the balance between activating and inhibiting receptors is crucial in immune responses. Increased expression of FcγRI and III on synovial macrophages of RA patients significantly correlated with a higher production of TNF-α and matrix metalloproteases (MMPs) \(^32\), which are crucial in enhancing joint destruction. Furthermore, several single-nucleotide polymorphisms (SNPs) in genes encoding the FcγRs have been associated with increased susceptibility to develop RA, underlining the importance of these receptors in RA pathogenesis \(^33\)-\(^35\). Some polymorphisms of FCGR2A and FCGR3A have been associated with an enhanced response to anti-TNF therapy \(^35\), \(^36\). These polymorphisms determine a low binding affinity for the Fc region of IgGs and may play an essential role via binding to the Fc region of anti-TNF agents, lowering their clearance from the circulation, thereby leading to a more effective therapeutic effect \(^36\). In mice, the deletion of various combinations of activating FcγRs significantly reduced both joint inflammation and destruction. In contrast, mice lacking the inhibiting FcγRIIb showed increased IC accumulation, resulting in increased inflammation and pathology \(^37\). In addition to increasing the production of pro-inflammatory and osteoclastogenic cytokines, ICs can also directly stimulate osteoclasts via binding to FcγRs expressed on their surface \(^38\). Upon IC stimulation the activating FcγRs signal through the γ-chain, thereby inducing differentiation of OCPs into osteoclasts via interacting with the ITAM costimulatory signaling both under physiologic and inflammatory conditions \(^39\). Further, FcγRs have been shown to modulate bone resorption in multiple experimental models of arthritis \(^38\)-\(^41\).
Collectively, these findings point out that these receptors and their balance play a crucial role in driving bone destruction during RA.

_FcγRs and S100A8/A9_

Activated macrophages are critical players during innate immune responses. They exert many effector functions, including the release of cytokines, reactive oxygen species (ROS), and matrix-degrading enzymes, as well as via phagocytosis and antigen presentation. FcγRs are highly present on activated macrophages and mediate many of their effector functions. Large amounts of ICs and pro-inflammatory cytokines present during RA strongly induce FcγR expression on macrophages. Alarmins S100A8 and S100A9 are cytosolic proteins forming the heterodimer S100A8/A9 that is released by stressed myeloid cells like granulocytes, monocytes and activated macrophages. The heterodimer is released during both the acute and chronic phase of joint inflammation and is among the most abundantly present pro-inflammatory proteins found in the synovium, synovial fluid, and serum of RA patients. In addition, S100A8/A9 levels positively correlate with the severity of joint inflammation and destruction in RA patients and it has been proposed as a marker to predict radiographic progression. A previous study found a significant positive association between the expression of activating FcγRI and IV in arthritic synovium and S100A8. Furthermore, _in vitro_ stimulation of macrophages with S100A8 skewed the balance towards the activating FcγRs. This shift makes macrophages more sensitive to IC stimulation, thereby resulting in increased pathology during experimental arthritis. In line with these findings, _S100a9−_/− mice, which additionally lack the systemic production of S100A8, showed decreased inflammation and bone pathology during experimental arthritis. Apart from having an indirect effect on joint damage via the regulation of inflammation, S100A8 also strongly stimulates osteoclast differentiation and activity, thus acting as a direct stimulator of bone destruction.

_RA and comorbidity_

As already introduced, RA is a systemic disease that particularly manifests within joints. The accumulation of ICs in the joints and the sustained production of chemotactic and pro-inflammatory factors trigger a positive feedback loop that perpetuates local inflammation. However, the underlying systemic disease often coincides with inflammation and other risk factors like insulin resistance and dyslipidemia (e.g. the metabolic syndrome) can also affect other organ systems like the periodontium, lungs, blood vessels and heart. These underlying systemic disease mechanisms could further promote joint destruction.
RA and periodontitis (PD) are both chronic inflammatory diseases sharing many features, particularly the production of pro-inflammatory cytokines like IL-1β, IL-6, TNF-α, and the presence of ACPA-ICs that strongly stimulate bone destruction, ultimately leading to focal erosions in the synovial joints and loss of the alveolar bone. Several studies have found an association between RA and PD. The incidence of PD was significantly increased in active RA patients as compared to healthy subjects, and vice versa patients with PD were more prone to develop RA than patients without PD. Oral bacteria responsible for the initiation and development of PD, and antibodies against them, were largely found both in serum and synovial fluid of RA patients throughout the disease. Further, antibiotics against bacterial infections showed to be effective in the treatment of RA, supporting the hypothesis of their involvement in RA pathogenesis.

Particularly IL-1β has been shown to drive tissue destruction in both RA and PD, likely due to its strong inducing effect on osteoclastogenesis and the consequent bone resorption. IL-1β strongly stimulates osteoclastogenesis and expansion of various subsets of OCPs. Moreover, recent findings showed that susceptibility to RA and PD was in part influenced by genetic variants of IL-1β, further suggesting an important role of IL-1β in the pathogenesis of both diseases. Two receptors, the type I IL-1 receptor (IL-1RI) and type II IL-1 receptor (IL-1RII) can bind IL-1β. Importantly, another protein, interleukin-1 receptor antagonist (IL-1Ra), also binds to IL-1RI, thereby inhibiting the IL-1 signaling and its effects on osteoclast formation and activity. Therefore, IL-1Ra deficient (Il1rn−/−) mice represent an ideal model to study the effects of enhanced IL-1 signaling on bone destruction and possible mechanisms underlying the enhanced bone destruction observed in RA and PD.

RA and the metabolic syndrome

The metabolic syndrome (MetS) is a chronic disorder that is often associated with increased risk of developing RA and cardiovascular diseases (CVDs). MetS is characterized by insulin resistance, reduced high-density lipoprotein cholesterol (HDL-C), increased low density lipoprotein cholesterol (LDL-C) and triglycerides, hyperglycemia, and high blood pressure. RA patients have a high incidence of various features of the MetS such as increased insulin resistance and dyslipidemia that is characterized by a strong reduction of HDL-C and increased LDL-C levels, thus increasing the occurrence of LDL oxidation to oxLDL. Together, these corresponding features may represent a link between RA and MetS. LDL-C hypercholesterolemia has been identified as a marker to predict radiographic progression in RA. However, the correlation
between LDL-C levels and RA progression is still a matter of debate. Of note, one study described that high LDL-C predispose to RA in women, but not in men, suggesting a role for sex hormones in the regulation of lipid effects on RA pathogenesis. Furthermore, lipid lowering therapies based on the use of statins were able to reduce the clinical effects of the MetS, and the risk of CVDs. However, controversial outcomes were found in RA patients undergoing statin treatment leaving the question whether dyslipidemia itself is to be considered detrimental with respect to the development of RA pathology unsolved.

**LDL oxidation in RA**

The transport of LDL from the liver to the other tissues in the body is mediated by apolipoproteins. Apolipoproteins, especially apolipoprotein B (APOB) and apolipoprotein E (APOE), are key in lipid transport as well as metabolism. LDL receptors (LDLr) present on the cells allow the uptake of LDL and tightly regulate intracellular levels of LDL. LDLr undergoes feedback regulation leading to a decrease of its synthesis after lipid uptake, thereby favoring the extracellular accumulation of LDL. In RA, the increased vascular permeability allows a higher influx of LDL in the inflamed joint tissues, where this LDL accumulates and gets oxidized (oxLDL) more easily due to the inflammation-driven ROS production. OxLDL is predominately taken up by macrophages via scavenger receptors CD36, SR-A, and LOX-1, and subsequently trafficked to lysosomes. OxLDL accumulation impairs lysosomal degradation, thus leading to the formation of massive cholesterol crystals. OxLDL is a potent stimulator of the innate immune response and its uptake by macrophages triggers a variety of pro- and anti-inflammatory responses. It can induce the expression of pro-inflammatory mediators such as IL-1, IL-6, S100A8/A9, monocyte chemoattractant protein (MCP-1), and IL-8, thereby potentiating the inflammatory response. However, oxLDL has been shown also to induce the production of TGF-β that has anti-inflammatory and anabolic capacities. Interestingly, a previous study showed that oxLDL stimulation of macrophages reduced the toll-like receptor 4 (TLR-4)-induced expression of pro-inflammatory genes in response to lipopolysaccharide (LPS), suggesting that uptake of oxLDL may serve as counteracting mechanism to interfere with the inflammatory response. Moreover, free oxLDL and anti-oxLDL antibodies forming oxLDL-ICs are internalized through FcγRs present on macrophages and as such can modulate the immune response.
Aim and outline of this thesis

The general purpose of this thesis was to broaden our knowledge of the relationship between the innate immune system and the development of joint pathology in experimental RA. Genetic and environmental factors lead to persistent activation of the innate immune system and are major factors involved in the etiopathogenesis of RA, thereby greatly contributing to bone destruction. However, the exact molecular mechanisms underlying the dysregulation of the innate immune system are not fully understood. Granulocytes, monocytes, macrophages, osteoclasts and their precursors are innate immune cells that play a central role in modulating inflammation-driven joint destruction. Therefore, further research would help to identify new therapeutic targets to dampen the innate response and possibly allow the treatment of earlier stages of RA pathology.

In Chapter 2, using IL-1Ra deficient (Il1rn−/−) mice, we studied the effects of an enhanced IL-1 signaling on different subsets of osteoclast precursors (OCPs), osteoclastogenesis and resorptive activity at different skeletal sites (long bone, jaw, vertebra, and calvaria) to deepen our understanding of the mechanistic link between RA and PD pathology.

As dysregulation of FcγR expression is often seen during RA and increased expression of activating FcγRs is associated with increased joint pathology, in Chapter 3, we investigated the role of FcγRs on bone destruction in experimental arthritis. Antigen-induced arthritis (AIA) was elicited locally in the knee joints of FcγRI,II,III,IV deficient (FcγRI,II,III,IV−/−) and their wild-type (WT) controls to assess inflammation and bone destruction. Next, to better characterize the role of FcγRIV on bone destruction, FcγRI,II,III,IV−/− mice were compared to FcγRI,II,III−/− controls.

In Chapter 4, we used Apoe−/− mice, which spontaneously develop high levels of LDL, to investigate the effects of hypercholesterolemia on the onset of inflammation and MMP-mediated cartilage destruction during AIA. As in the arthritic environment high LDL levels are oxidized to oxLDL, we investigated in vitro the ability of oxLDL to modulate FcγR expression on macrophages, which is crucial to the onset and progression of arthritis. In Chapter 5, we investigated more in detail the effects of high LDL levels on bone destruction during experimental arthritis. Since the increased oxidation in the arthritic environment could affect osteoclast formation and their resorptive activity, we studied in vitro the effects of LDL/oxLDL on the osteoclast differentiation and function. Moreover, we assessed the involvement of LDL/oxLDL in the modulation of the ITAM-costimulatory pathway of osteoclastogenesis to explore potential mechanisms.

In this thesis we used Apoe−/− mice predominantly to explore the effects of high LDL levels and their oxidation to oxLDL during experimental arthritis. However, apart from having a role in lipid metabolism, APOE has been shown to modulate the functions of various
immune cells and may be involved in the pathogenesis of other rheumatic diseases like inflammatory osteoarthritis (OA). Interestingly, APOE shows anti-inflammatory effects on macrophages. In humans there are three isoforms of APOE (APOE-ɛ2, ɛ3, ɛ4), which have been associated with a different extent of anti-inflammatory capacity. Therefore, in Chapter 6 we induced experimental inflammatory osteoarthritis (collagenase-induced OA (CiOA)) in mice in which the murine Apoe gene is replaced by human APOE-ɛ3 or APOE-ɛ4 gene and explored the possibility that different genotypes of APOE would affect differently OA pathology.

In Chapter 7 we described a novel assay for studying osteoclastogenesis and bone erosion using the immortalized ER-Hoxb8 myeloid precursors cell line. Moreover, we demonstrated that it is possible to generate genetically modified precursors using the CRISPR/Cas9 technology to allow functional studies of proteins involved in osteoclastogenesis and resorptive activity.

Finally, in Chapter 8 we summarized the results presented in thesis and discussed about possible implications of these findings and future perspectives.
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Enhanced IL-1 signaling in vivo enhances osteoclast function at different skeletal sites, particularly in long bone and jaw marrow, by increasing the number of monocytic osteoclast precursors

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Manuscript in preparation
Abstract

**Background:** It is commonly accepted that the phenotype of osteoclasts varies at different skeletal locations. This variation in osteoclast phenotype likely arises from a differential local priming or a skewed distribution of the diverse osteoclast precursors (OCPs). We recently showed that interleukin-1β (IL-1β) has diverse stimulatory effects on different murine long bone marrow OCPs *in vitro*.

**Objective:** In the present study, we investigated the *in vivo* role of enhanced IL-1 signaling prior to development of inflammation and bone erosion on the composition of three different OCP populations at four different bone locations, their potential to differentiate into osteoclasts and resorption *in vitro*.

**Methods:** Interleukin-1 receptor antagonist knockout deficient (*Il1rn*<sup>-/-</sup>) Balb/c and wildtype (WT) Balb/c mice of 14-16 weeks of age were compared to investigate how enhanced IL-1 signaling affects the marrow composition of OCPs and their osteoclastogenic potential in long bone, calvaria, vertebra and jaw. Cell infiltration and bone erosions were assessed by histology and micro-computed tomography (µCT), respectively. From these bones, bone marrow (BM) cells were isolated and labeled with anti-CD31 and Ly-6C. The percentage of osteoclast precursors (early blasts (CD31<sup>hi</sup> Ly-6C<sup>-</sup>), myeloid blasts (CD31<sup>+</sup> Ly-6C<sup>+</sup>) and monocytes (CD31<sup>-</sup> Ly-6C<sup>hi</sup>) was assessed by flow cytometry. Next, BM cells from these four skeletal sites were cultured with macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) on bone or on hydroxyapatite-coated plates to assess their osteoclastogenic potential and monitor their resorption ability.

**Results:** At the time-point of cell isolation, *Il1rn*<sup>-/-</sup> mice did not yet show any sign of inflammation or bone destruction as determined by histological and µCT analysis. However, despite this lack in inflammation or destruction, *Il1rn*<sup>-/-</sup> mice had a higher percentage OCPs in long bone and jaw BM when compared to WT Balb/c. At both locations, particularly the monocyte subset was strongly induced, while the pool of myeloid blasts was increased only in long bone. In contrast, in vertebrae and calvaria we did not observe any change in the various OCP subsets between the two strains. The *in vitro* osteoclastogenic potential of cells isolated from long bone, calvaria and jaw was increased in the absence of IL-1Ra, resulting in higher osteoclast number and size. Whereas osteoclastogenesis was not increased in OCPs derived from vertebrae of *Il1rn*<sup>-/-</sup> mice, *in vitro* mineral dissolution was boosted in all skeletal sites as compared to WT osteoclasts.

**Conclusion:** Enhanced IL-1 signaling *in vivo* increases osteoclast function at different skeletal sites by boosting the number of the monocytic subset of OCPs in long bone and jaw, probably underlying the formation of large osteoclasts and increased resorptive activity at those bone sites, which are favored sites of inflammation in rheumatoid arthritis (RA) and periodontitis (PD).
CHAPTER 2

Introduction

Osteoclasts are multinucleated bone-resorbing cells that are essential for the homeostasis of bone. Shifting the balance in bone turnover, results in excessive bone destruction in diseases like rheumatoid arthritis (RA), osteoporosis (OP) and periodontitis (PD). Various studies showed a correlation between RA and the development of PD. It has been reported that patients with active RA have a significantly increased chance of developing PD when compared with healthy controls and that patients with PD have a higher incidence of RA than patients without PD. Both diseases are associated with inflammation and high levels of pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α). Particularly IL-1β has been shown to strongly stimulate osteoclastogenesis and bone resorption. IL-1β can bind to two receptors, type I IL-1 receptor (IL-1RI) and type II IL-1 receptor (IL-1RII). The endogenous inhibitor IL-1 receptor antagonist (IL-1Ra) competitively blocks the binding of IL-1 β to IL-1RI, thereby regulating its signaling. Another inhibitory mechanism is mediated by IL-1RII, which exists both as membrane-bound and soluble receptor. In contrast to IL-1RI, IL-1RII lacks the Toll/IL-1R domain at the cytoplasmic terminus and is therefore incapable of signaling. IL-1Ra counteracts IL-1 effects on bone destruction by negatively regulating osteoclastogenesis as well as bone resorption. Mice lacking the encoding gene for IL-1Ra (Il1rn) represent an ideal model to study enhanced IL-1 signaling on bone destruction. Il1rn deficient (Il1rn−/−) mice have been shown to develop bone erosion at various skeletal sites, particularly the ankle joint and the jaw. Upon aging, Il1rn−/− mice spontaneously develop inflammation in the ankle joint, showing synovial and peri-articular inflammation, cell infiltration and articular erosions. In contrast, spine and knee joints are less susceptible to develop local inflammation. Previous studies showed that various mononuclear cell fractions isolated from mouse BM differ in their capacity to form osteoclasts and their response to pro-inflammatory cytokines, suggesting that dysregulation of IL-1 signaling can have diverse effects depending on the skeletal sites and their susceptibility to develop inflammation. Many studies found that osteoclasts isolated or differentiated from bone marrow cells of various skeletal sites are not always identical. The bone marrow of each skeletal site contains different subsets of osteoclast precursors (OCPs) based on their surface markers, recognized as early blasts (CD31hi Ly-6C−), myeloid blasts (CD31 Ly-6C+), and monocytes (CD31 Ly-6Chi) 22. These three subsets from bone marrow have the capacity to differentiate into osteoclasts, but they respond differently to macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) and to IL-1β with respect to proliferation, multinucleation, life span, and bone resorption. In a previous study it was shown that IL-1β stimulates proliferation of early blasts, while it induces multinucleation by all the
subsets, being most pronounced in myeloid blast cultures. Therefore, we hypothesized that enhanced IL-1 signaling drives the number and the osteoclastogenic potential of OCPs differently at various skeletal sites. In the present study, using I{\textsuperscript{Il1rn}}{\textsuperscript{-/-}} mice, we aimed to elucidate the effects of enhanced IL-1 signaling on the number and characteristics of OCPs in the bone marrow of long bone, calvaria, vertebra and jaw and their potential in osteoclastogenesis and resorptive activity of bone.

**Methods**

**I{\textsuperscript{Il1rn}}{\textsuperscript{-/-}} mice**

I{\textsuperscript{Il1rn}}{\textsuperscript{-/-}} mice on Balb/c background were kindly supplied by Dr. M. Nicklin (Sheffield, UK) and generated as previously described. The mice were housed in conventional filter-top cages, and food and water were provided ad libitum. Age-matched Balb/c mice were used for all experiments. All animal studies were approved by the institutional animal ethics committees from the Vrije Universiteit (VU), Amsterdam, the Netherlands and the Radboud university medical center (Radboudumc), Nijmegen, the Netherlands.

**Histology**

All specimens from wild type (WT) and I{\textsuperscript{Il1rn}}{\textsuperscript{-/-}} mice were fixed in 4% paraformaldehyde (PFA), decalcified in ethylenediaminetetraacetic acid (EDTA) and subsequently embedded in paraffin wax. Longitudinal sections of 7 μm were stained with hematoxylin & eosin (H&E) for qualitative assessment of inflammation, defined as cell infiltration.

**Micro-computed tomography (μCT)**

Femurs, calvariae, vertebrae and jaws were dissected from WT or I{\textsuperscript{Il1rn}}{\textsuperscript{-/-}} mice and fixed with 4% PFA for 48 hours, then stored in 70% ethanol at 4°C. Scans were obtained with a SCANCO Medical machine and ex-vivo μCT analysis were performed with Skyscan 1172 Software (Bruker, Germany). The region of interest (ROI) was set using a manually determined global threshold. 3D microstructural bone properties, including bone volume fraction (BV/TV), specific bone surface (BS/BV), trabecular thickness (Tb.Th.), and trabecular separation (Tb.Sp.), were calculated according to the manufacturer’s software.
CHAPTER 2

**Bone marrow isolation**

Il1rn<sup>-/-</sup> mice and Balb/c WT male mice were sacrificed between 14-16 weeks of age and only a specific subset of mice that did not show any signs of macroscopic inflammation were further analyzed. BM cells were isolated from four skeletal sites: long bone, calvaria, vertebra and jaw. Soft tissue was removed from the bones followed by hard mashing in a mortar with 5 ml α-MEM (Gibco; Thermo Fisher Scientific, Paisley, Scotland) supplemented with 5% fetal calf serum (HyClone; GE Healthcare Life Sciences, Logan, UT) and 1% penicillin-streptomycin-fungizone (Sigma-Aldrich, St. Louis, MO). The released cells were aspirated through a 21-gauge needle and the suspended bone marrow cells were filtered through a 40 μm filter. The number of cells were counted using a MUSE<sup>TM</sup> cell analyzer (Merck, Germany).

**Immunofluorescence labeling and flow cytometric analysis**

First, bone marrow (BM) cells were incubated with Fc-blocking antibody (BD Pharmingen antimouse CD16/CD32, clone 2.4G2; BD Biosciences, San Jose, CA, USA), followed by staining with CD31 (AbD Serotec, Kidlington, United Kingdom) diluted in FACS buffer (1% albumin from BSA [Sigma-Aldrich]). After 30 min, samples were incubated with Ly-6C (AbD Serotec, Kidlington, United Kingdom) diluted in FACS buffer containing streptavidin PE (Becton Dickinson, San Jose, CA). Cell viability was discriminated using SYTOX blue viability dye and the percentage of early blasts (CD31<sup>hi</sup> Ly-6C<sup>-</sup>), myeloid blasts (CD31<sup>+</sup> Ly-6C<sup>+</sup>), and monocytes (CD31<sup>-</sup> Ly-6C<sup>hi</sup>) were measured by Gallios Flow Cytometer. Analyses were performed using the Kaluza Analysis software 1.3 (Beckman Coulter, Brea, CA).

**Cell culture**

Bone marrow cells isolated from all four skeletal sites were seeded in 96-well plates (Cellstar; Greiner Bio-One, Monroe, NC) and cultured in α-MEM medium containing 30 ng/ml M-CSF (R&D Systems, Minneapolis, MN) and 20 ng/ml RANKL (RANKL-TEC, R&D systems). Culture media were refreshed after 3 days, and cultures were stopped after 6 days and fixed in 4% formaldehyde for tartrate resistant acid phosphatase (TRAcP) staining, or lysed in RNA lysis buffer (Qiagen, Hilden, Germany) for RT-PCR.

**Analysis of tartrate-resistant acid phosphatase positive cells**

Cells cultured on bone slices were fixed and stained for TRAcP using a commercially available leucocyte acid phosphatase kit (Sigma-Aldrich). The staining procedure was performed following the manufacturer's instructions and has been described previously<sup>5</sup>. Nuclei were counterstained by 4’6-diamidino-2-phenylindole (DAPI), and the number of TRAcP positive (TRAcP<sup>+</sup>) cells with three or more nuclei was assessed and categorized into four groups: 3-5, 6-10, 11-20 and >20 nuclei. The number of each
category was counted using a combination of light and fluorescence microscopy (Leica DFC320; Leica Microsystems, Wetzlar, Germany). Counts were expressed as osteoclast number per cm².

**Biomimetic hydroxyapatite coating and analysis of resorption areas**

Biomimetic hydroxyapatite-coated 96-well plates were prepared as previously described. This coating resembles the mineral part of bone tissue. The coated 96-well plates were sterilized by UV light exposure. The cultures were fixed in 4% paraformaldehyde (PFA) after 8 days and the resorbed area in relation to the total coating area was visualized by light microscopy (Leica DFC320), and quantified using Image Pro Plus (Media Cybernetics, Silver Spring, MD).

**Quantitative RT-PCR**

The mRNA expression of Il1rn, the encoding gene for IL-1Ra, was measured by RT-PCR as previously described. Samples were normalized for the expression of the housekeeping gene B2m encoding for Beta 2 Microglobulin. Primer sequences used were the following: for mouse B2m, Forward: TGCTATCCAGAAAACCCCTCAA; Reverse: GCGGTTGGAACGTGTGTTACG (Accession number: ENSMUSG00000060802); for mouse Il1rn, Forward: TGTGCCAAGTCTGGAGATGATC; Reverse: TTGTCTTGGCTACGATG (Accession number: ENSMUST00000114487). The relative expression of each gene was calculated as $2^{-\Delta Ct}$, $\Delta Ct = (Ct_{\text{gene of interest}} - Ct_{\text{B2m}})$ and the results were shown as fold increase.

**Statistical analysis**

Comparison between wild type and IL-1Ra−/− mice were tested by Student’s t-test using GraphPad Prism (version 6.00; GraphPad Software, LaJolla, CA). Data are expressed as mean ± SD. One-way ANOVA followed by Tukey-Kramer’s multiple comparison test was used for three or more comparisons. P<0.05 was considered as a significant difference.

**Results**

*Enhanced IL-1 signaling significantly increases the number of OCPs, particularly monocytes in the long bone and jaw*

We used a subset of Il1rn−/− mice that virtually had developed no inflammation yet as determined by macroscopic score (data not shown) of the ankle joints. Histological assessment of all four skeletal locations (long bone, calvaria, vertebra, and jaw) indeed showed absence of inflammatory cells and bone erosion (Figure S1). In line with this, *ex vivo* µCT scans showed similar BV/TV ratio in the trabecular region in the various skeletal sites in WT and Il1rn−/− mice. Furthermore, BS/BV, Tb. Th. and Tb. Sp. as other
parameters for analysis of trabecular bone were similar between WT and \( \text{iIL1rn}^{+/}\) specimens (Figure 1A-D), suggesting that bone destruction did not occur yet.

Figure 1. microCT analysis of WT and \( \text{iIL1rn}^{+/}\) mice showed no differences in the bone microstructure independently of the skeletal site. To assess bone destruction at the different skeletal sites various 3D microstructural bone properties, including bone volume fraction (BV/TV), specific bone surface (BS/BV), trabecular thickness (Tb.Th.), and trabecular separation (Tb.Sp.) were measured in all specimens from \( \text{iIL1rn}^{+/}\) mice and wild type (WT) controls (A-D) \((n=3\) mice/group). These parameters were comparable between the two mouse strains irrespective of the bone site, suggesting that these \( \text{iIL1rn}^{+/} \) mice did not develop structural pathology yet.
After we established the absence of structural pathology in these \( \text{Il1rn}^{-/-} \) mice that did not develop inflammation, we explored whether enhanced IL-1 signaling affected the different OCP subsets in the BM (early blasts, myeloid blasts and monocytes) using flow cytometry (gating strategy shown in Figure S2). The most abundant increase of the three subsets was observed in the long bone of \( \text{Il1rn}^{-/-} \) mice, where particularly the percentage of monocytes was strongly increased (from 2.0% in WT to 4.4% in \( \text{Il1rn}^{-/-} \) mice) (Figure 2A), as well as that of myeloid blasts, (increase from 0.7% in WT to 1.2% in \( \text{Il1rn}^{-/-} \) mice). Interestingly, also the jaw marrow showed a 2-fold increase of the monocyte subset (from 0.4% in WT to 0.8% in \( \text{Il1rn}^{-/-} \) mice) (Figure 2D). In contrast to long bone and jaw no differences in percentual contribution were found in any of the OCP pools between WT and \( \text{Il1rn}^{-/-} \) mice in calvaria (Figure 2B) and vertebrae (Figure 2C).

**Enhanced osteoclast formation and multinucleation in bone marrow cells derived from long bone and jaw, but also calvaria**

We next investigated whether enhanced IL-1 signaling in vivo altered the potential to induce in vitro osteoclast differentiation on bone. The whole population of bone marrow (BM) cells from the four skeletal sites was seeded on bone slices and cultured for 6 days for osteoclast differentiation using optimal concentrations of M-CSF and RANKL. All cultures of WT osteoclasts expressed \( \text{Il1rn} \), whereas as expected no expression was detected in \( \text{Il1rn}^{-/-} \) osteoclasts obtained from any of the skeletal sites (Figure S3). OCPs present in BM obtained from WT and \( \text{Il1rn}^{-/-} \) specimens were all able to differentiate efficiently into multinucleated and TRAcP\(^+\) osteoclasts (Figure 3A). Next, osteoclasts were classified depending on the number of their nuclei (3-5; 6-10; 11-20 and >20), that positively correlated with osteoclast size and activity. \( \text{Il1rn}^{-/-} \) BM cells from long bone, jaw and calvaria showed a significantly increased osteoclastogenesis as compared to their WT controls. (Figure 3B, C, E). Particularly, \( \text{Il1rn}^{-/-} \) cells from long bone and jaw gave rise to a higher number (2- and 3-fold higher, respectively) of intermediate and large osteoclasts (>5 nuclei and >10 nuclei, respectively), whereas the enhanced osteoclastogenesis in \( \text{Il1rn}^{-/-} \) cells from calvaria (4 times higher as compared to WT control cells) was mainly due to the increase of small (3-5 nuclei) and intermediate (>5 nuclei) osteoclasts. In contrast, a comparable number of osteoclasts from vertebral BM of WT and \( \text{Il1rn}^{-/-} \) mice was observed.
Figure 2. Absence of IL-1Ra increases the percentage of myeloid blasts in long bone, and the percentage of monocytes in long bone and jaw. Percentage of early blasts, myeloid blasts and monocytes from long bone (A), calvaria (B), vertebra (C) and jaw (D) were quantified, and compared between wild type (WT) (black column) and Il1rn−/− (white column) mice (n=6, *P<0.05, ***P<0.001). Whereas calvaria and vertebral bone marrow (BM) of Il1rn−/− mice showed no differences in the percentage of various osteoclast precursor (OCP) subsets, we found a significant increase of both myeloid blasts and monocyte subsets in the BM of long bone. Similarly, the monocyte subset of OCPs was also significantly increased in the jaw BM of Il1rn−/− mice as compared to WT controls.
Figure 3. Bone marrow cells from Il1rn<sup>−/−</sup> mice showed increased osteoclastogenic capacity in long bone, calvaria and jaw, but not vertebra. A) Microphotographs of TRAcP<sup>+</sup> multinucleated cells formed on bone slices. Bone marrow cells from long bone, calvaria, vertebra and jaw were cultured with 30 ng/ml M-CSF and 20 ng/ml RANKL on bone slices for 6 days. Osteoclasts were stained by TRAcP (purple) and nuclei were counterstained by DAPI (blue). The number of osteoclasts (>2 nuclei) was counted for long bone (A), calvaria (B), vertebra (C) and jaw (D) and compared between wild type (WT) and Il1rn<sup>−/−</sup> mice. Osteoclastogenesis was significantly higher in long bone, calvaria, and jaw of Il1rn<sup>−/−</sup> osteoclast precursors (OCPs) as compared to WT OCPs. In contrast, WT and Il1rn<sup>−/−</sup> OCPs isolated from vertebrae formed a comparable number of osteoclasts. Scale bar= 100 µm. (n=6, *P<0.05, **P<0.01, ***P<0.001).

Il1rn<sup>−/−</sup> osteoclasts show increased in vitro resorption on biomimetic hydroxyapatite-coated plates independently of the skeletal site

As osteoclasts can react differently depending on the bone surface we seeded the BM cells from the four skeletal sites on biomimetic hydroxyapatite-coated plates to determine whether the TRAcP<sup>+</sup> multinucleated cells were functional osteoclasts. The absence of Il1rn resulted in increased resorption from all skeletal sites, as indicated by larger pit areas devoid of mineral as compared to WT control osteoclasts (Figure 4A-D). Quantification of resorptive activity showed a sharp increase of the eroded areas (~8-fold higher). Together, these findings suggest that under optimal concentration of M-CSF and RANKL the enhanced IL1 signaling in Il1rn<sup>−/−</sup> osteoclasts significantly increases the breakdown of hydroxyapatite independently of the skeletal site they were isolate from.
Discussion

The present study showed that the effects of higher IL-1 signaling on BM OCPs before onset of inflammation and bone erosion were site-specific. In particular, the relative contribution of the monocyte subset in the OCP population was increased in the BM of long bone and jaw when compared to calvaria and vertebra. Further, we showed that enhanced IL-1 signaling significantly increased the in vitro formation of osteoclasts and resorption irrespective of the site of isolation.

Il1rn−/− mice are commonly used as a model for studying bone loss during inflammatory arthritis, however little is known about how osteoclastogenesis and resorptive activity is regulated at different skeletal sites prior to onset of inflammation and bone destruction.
In this study, we used a subset of mice that showed no signs of inflammation or bone destruction in any of the investigated skeletal sites. Il1rnull mice have been shown to develop clear inflammation at some preferential skeletal sites like the ankle and jaw, as well as in the vertebrae in ageing mice. Furthermore, as both osteoclastogenesis and bone resorption are highly regulated by inflammation we investigated OCPs under enhanced IL-1 signaling at different skeletal sites prior to the development of macroscopic inflammation.

It is known that pro-inflammatory cytokines such as TNF-α can expand the OCPs in inflammatory RA models. However, little is known about the effects of IL-1 on the expansion of various OCPs in vivo. In this study, enhanced IL-1 signaling resulted in a higher percentage of myeloid blasts and particularly monocytes in the BM of long bone and jaw. The latter are the most recruited OCP subset during inflammation and have a longer lifespan as compared to the other OCPs. Monocytes and polymorphonuclear cells (PMNs) are potent producers of pro-inflammatory cytokines that can boost bone destruction during inflammatory diseases characterized by excessive bone destruction. In a previous study it was shown that among the various OCP subsets, particularly myeloid blasts give rise to large osteoclasts when stimulated by IL-1β. Therefore, the increased percentage of myeloid blasts observed in long bone BM, and monocytes in both long bone and jaw BM in conjunction with enhanced IL-1 signaling may lead to increased osteoclast formation and resorption at those bone sites when local inflammation is present. These findings suggest that IL-1 is not only able to activate the precursors via triggering the osteoclastogenic signaling pathway, but that can also site-specifically steer the composition of the OCP pools towards the monocyte subset. However, as IL-1β has clonogenic effects on various cell types, thus inducing neutrophilia, leukocytosis and thrombocytosis in vivo, it may be that enhanced IL-1 signaling stimulated the proliferation of these specific OCP subsets. Apart from IL-1Ra, the expression of IL-1R1 and IL-1R2 is also important to modulate IL-1 signaling and the consequent inflammatory response. IL-1R2, either in the membrane-bound and soluble form, acts as decoy receptor for IL-1R1, thereby preventing IL-1 binding and further potentiating IL-1Ra inhibiting effects. Therefore, the enhanced IL-1 signaling may increase the expression of IL-1R1 on OCPs and as such contribute to activate osteoclasts and enhance bone destruction. Moreover, the IL-1-driven induction of other pro-inflammatory cytokines such as TNF-α activates signaling pathways that differently regulate bone destruction at the various skeletal sites. Together, all these factors can lead to an increased number of osteoclasts and can explain the increased susceptibility of Il1rnull mice to develop focal erosions in the long bones and jaw.
Further, this can represent a mechanism underlying bone inflammation-driven bone destruction in RA and PD. Several studies have shown that inflammation drives bone destruction. In particular, previous studies have shown a strong correlation between active RA, characterized by high grade inflammation and joint destruction, and progression of PD. Protein citrullination, the composition of the subgingival microbiome, and systemic inflammation are factors that have been described to link RA to PD pathology. The production of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 in both synovial and periodontal tissues triggers similar immune responses which drive synovial and periodontal inflammation, ultimately leading to joint destruction and tooth bone loss. Together, our findings indicate that enhanced IL-1 signaling site-specifically increases the monocyte subset of OCPs and support the hypothesis that local differences in OCPs and their response to cytokines can exacerbate bone destruction in long bone and jaw, thus representing an additional mechanistic link between RA and PD.

In contrast to long bone and jaw, enhanced IL-1 signaling did not alter the composition and percentage of OCPs in vertebral and calvaria BM. As we find that deletion of Il1rn boosted the *in vitro* mineral dissolution, this suggests that an enhanced IL-1 signaling together with the use of optimal concentrations of M-CSF and RANKL used to induce osteoclastogenesis make the OCPs more active, overruling the local differences present *in vivo*. Therefore, additional *in vitro* studies using suboptimal concentrations of M-CSF and RANKL would be helpful to determine the osteoclastogenic potential of OCPs at the various skeletal locations. Enhanced bone destruction in the vertebrae is mainly observed in age-related and metabolic disorders such as OP. As systemic inflammation is associated with an increased risk of developing OP in older subjects it may be that enhanced IL-1 signaling increases osteoclast activity in the vertebrae only in combination with ageing, probably explaining the lack of structural pathology observed in the vertebrae of Il1rn−/− mice. Bone destruction in the skull predominantly occurs in rare skeletal disorders like Paget’s diseases, where the disruption of bone metabolism rather than inflammation is the central feature, or after an injury as part of the healing process. It was previously shown that osteoclasts from long bone and calvaria use different proteolytic enzymes to digest the bone matrix, they make use of another ion transporter to dissolve bone minerals. Furthermore, a previous study showed that long bone marrow has a higher osteoclastogenic potential as compared to cells from the jaw marrow. In addition, we propose that OCPs present at different skeletal locations may give rise to phenotypically different osteoclasts, probably explaining the lower susceptibility of Il1rn−/− mice to develop erosions in the
spine \textsuperscript{14} and the lack of enhanced \textit{in vivo} resorption of the calvarial bone in absence of an inflammatory trigger \textsuperscript{48}.

We hypothesized that different skeletal locations might follow different mechanisms of osteoclastogenesis in case of excessive IL-1 signaling. This study provides new insights on osteoclast differentiation of the BM cells from different skeletal sites induced by the absence of IL-1Ra and further highlights the diversity of IL-1 signaling regulation throughout the body under physiological state. Collectively, our data suggest that sustained IL-1 signaling by deletion of IL-1Ra elicited a skeletal site-dependent response in bone marrow composition of long bone and jaw and this can represent an additional link between RA and PD pathology.

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References


Figure S1. Comparable cell infiltration in WT and \( \text{Il1rn}^{-/-} \) specimens. Representative microphotographs of long bone (A), calvaria (B), vertebra (C), and jaw (D) in WT and \( \text{Il1rn}^{-/-} \) mice (\( n=3 \) mice/group). Qualitative assessment of cell infiltration (black arrows) as parameter for tissue inflammation was performed in mice that showed no signs of macroscopic inflammation. In long bone cell infiltration we assessed at the articulating surface of the joint (A) and at the transsuture in the calvaria bones (B). In the vertebrae, cell infiltration was assessed in the distal part of the vertebral body in close proximity to the cartilage tissue (C). In the jaw cell infiltration was determined in the gum junctional epithelium (D). In all skeletal sites no differences in cell infiltration within the synovial tissue were found. Original magnification 100x. \( F = \) femur; \( T = \) tibia; \( TS = \) transsuture; \( VB = \) vertebral body; \( GJE = \) gum junctional epithelium.
Figure S2. Gating strategy for the cytofluorimetric analysis of BM OCPs from long bone, calvaria, vertebra and jaw in WT and Il1rn−/− mice. Cells were labeled by anti-CD31 and Ly-6C and osteoclast precursor subsets were gated as: early blasts (CD31^hi Ly-6C^−), myeloid blasts (CD31^+ Ly-6C^+), and monocytes (CD31^− Ly-6C^hi) as circled in each profile. Other populations mainly contained lymphocytes (Labeled A), erythroid blasts (Labeled B) and granulocytes (Labeled C).

Figure S3. Il1rn−/− osteoclasts lack the expression of Il1rn. Gene expression of Il1rn was measured in WT and Il1rn−/− osteoclasts derived from long bone, calvaria, vertebra, and jaw BM. Gene expression was normalized for the expression of B2m as reference gene.
CHAPTER 3

Fcγ receptor-mediated influx of S100A8/A9-producing neutrophils as inducer of bone erosion during antigen-induced arthritis


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Abstract

**Background:** Osteoclast-mediated bone erosion is a central feature of rheumatoid arthritis (RA). Immune complexes, present in a large percentage of patients, bind to Fcγ receptors (FcγRs), thereby modulating the activity of immune cells. In this study, we investigated the contribution of FcγRs, and FcγRIV in particular, during antigen-induced arthritis (AIA).

**Methods:** AIA was induced in knee joints of wild-type (WT), FcγRI,II,III⁻/⁻, and FcγRI,II,III,IV⁻/⁻ mice. Bone destruction, numbers of tartrate-resistant acid phosphatase-positive (TRAP⁺) osteoclasts, and inflammation were evaluated using histology; expression of the macrophage marker F4/80, neutrophil marker NIMP.R14, and alarmin S100A8 was evaluated using immunohistochemistry. The percentage of osteoclast precursors in the bone marrow was determined using flow cytometry. In vitro osteoclastogenesis was evaluated with TRAP staining, and gene expression was assessed using real-time PCR.

**Results:** FcγRI,II,III,IV⁻/⁻ mice showed decreased bone erosion compared with WT mice during AIA, whereas both the humoral and cellular immune responses against methylated bovine serum albumin were not impaired in FcγRI,II,III,IV⁻/⁻ mice. The percentage of osteoclast precursors in the bone marrow of arthritic mice and their ability to differentiate into osteoclasts in vitro were comparable between FcγRI,II,III,IV⁻/⁻ and WT mice. In line with these observations, numbers of TRAP⁺ osteoclasts on the bone surface during AIA were comparable between the two groups. Inflammation, a process that strongly activates osteoclast activity, was reduced in FcγRI,II,III,IV⁻/⁻ mice, and of note, mainly decreased numbers of neutrophils were present in the joint. In contrast to FcγRI,II,III,IV⁻/⁻ mice, AIA induction in knee joints of FcγRI,II,III⁻/⁻ mice resulted in increased bone erosion, inflammation, and numbers of neutrophils, suggesting a crucial role for FcγRIV in the joint pathology by the recruitment of neutrophils. Finally, significant correlations were found between bone erosion and the number of neutrophils present in the joint as well as between bone erosion and the number of S100A8-positive cells, with S100A8 being an alarmin strongly produced by neutrophils that stimulates osteoclast resorbing activity.

**Conclusions:** FcγRs play a crucial role in the development of bone erosion during AIA by inducing inflammation. In particular, FcγRIV mediates bone erosion in AIA by inducing the influx of S100A8/A9-producing neutrophils into the arthritic joint.
Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease that primarily affects the joints. Along with inflammation, excessive bone erosion is one of the central hallmarks of this disease. Next to generalized osteoporosis, severe focal bone erosions are observed at the interface between the inflamed synovium and the bone. Osteoclasts, which differentiate from myeloid precursor cells under the influence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), are the cells responsible for this deleterious process. Therefore, a deeper understanding of how the inflammatory response increases bone erosion in this disease is likely to be helpful in identifying new therapeutic targets.

Autoantibodies such as rheumatoid factor (RF) and anticitrullinated protein antibodies are present in the serum and synovial fluid of a large percentage of patients with RA. Of note, in patients with RA the presence of autoantibodies predates disease onset and correlates with disease progression and severity. Immunoglobulin G (IgG) antibodies can form immune complexes (ICs) with their cognate antigens and subsequently bind to Fcγ receptors (FcγRs), thereby modulating the activity of the FcγR-bearing immune cells.

In mice, four different FcγRs have been identified, of which the activating FcγRI, FcγRIII, and FcγRIV stimulate the cell via the activation motif immunoreceptor tyrosine-based activation motif (ITAM), leading to effector functions such as phagocytosis, antigen presentation, and cytokine secretion. In contrast, FcγRIIb is an inhibitory receptor, and its intracellular domain contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that counteracts the signaling of the activating FcγRs. Alterations in the expression of FcγRs have been described in circulating monocytes and synovial tissue of patients with RA, suggesting their involvement in the pathogenesis of RA. Moreover, the crucial pathogenic role of FcγRs has been proven in a multitude of experimental arthritis models, such as collagen-induced arthritis (CIA), glucose-6-phosphate isomerase-induced arthritis, collagen type II antibody-induced arthritis, the K/B×N serum transfer model, IC arthritis, and antigen-induced arthritis (AIA) models. Overall, despite some differences between the various experimental models, activating FcγRs stimulate innate immune cells, leading to deleterious effects. On the contrary, FcγRIIb induces negative feedback in the production of autoantibodies, thereby protecting the joint from the development and progression of the disease. However, the function of the various FcγRs and their exact mechanism of action in the modulation of bone erosion remain to be elucidated.
In the AIA experimental RA model, the injection of methylated bovine serum albumin (mBSA) into the knee joints of previously immunized mice results in a strong local accumulation of ICs that, via activation of the immune system, are responsible for the degradation of both bone and cartilage. In previous studies using this model, we determined the relationship between synovial inflammation and bone destruction using knockout mouse strains for various (combinations of) FcγRs. We found that there was a link between FcγR-mediated inflammation and bone erosion. Whereas FcγRs are expressed on osteoclasts and may thus be involved in their differentiation and activation, a central role in IC-mediated inflammation has been attributed to the FcγR-mediated activation of macrophages during AIA. Their IC-mediated activation leads to the production of a plethora of mediators, such as chemotactic factors, responsible for the recruitment of, among others, neutrophils into the joint. However, which FcγR is particularly involved in regulating this cell influx and which cell is dominant in mediating bone destruction is still a matter of debate.

The importance of neutrophils in arthritis development has been shown in the K/B×N serum transfer experimental RA model, in which depletion of neutrophils leads to complete protection from disease development. In agreement with this finding, high numbers of neutrophils are present in the joints of patients with active RA. Two factors produced by neutrophils in high quantities are the alarmins S100A8 and S100A9, which make up roughly 40% of all cytosolic proteins. S100A8/A9 are small calcium-binding proteins that, upon cell stress, are released into the extracellular environment, where they function as potent inducers of the immune system. High levels of S100A8/A9 are present in the synovial fluid of patients with RA. Moreover, it has been shown that S100A8 is able to directly stimulate osteoclast activity via TLR4, suggesting a possible mechanism through which the IC-activated innate immunity can regulate bone erosion in RA.

In the present work, we investigated the involvement of FcγRs, and of FcγRIV in particular, in the regulation of osteoclast-mediated bone resorption. We induced AIA in mice deficient in all four FcγRs (FcγRI,II,III,IV-/- mice) and in their wild-type (WT) controls. The role of FcγRIV in particular was studied by comparing the development of AIA in FcγRI,II,III,IV-/- and FcγRI,II,III-/- mice.
Methods

Animals

FcγRI,II,III,IV-/- mice in a C57BL/6 background were developed by Dr. S. Verbeek (Leiden University Medical Center, Leiden, the Netherlands) (Dr. J. Sjef Verbeek personal communication, January 2016). Control C57BL/6 mice were purchased from Janvier Labs (Le Genest Saint Isle, France). FcγRI,II,III-/- mice and their controls were generated as previously described 29. Mice were housed under standard conditions in filter-top cages and fed a standard diet with food and tap water ad libitum. All animal studies were carried out according to the Dutch law and approved by the local animal experimentation committee (RU-DEC 2012-209).

Induction of AIA

Mice were immunized with 100 μg of mBSA (Sigma-Aldrich, St. Louis, MO, USA) emulsified in complete Freund’s adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). Heat-killed Bordetella pertussis was administered intraperitoneally as an additional adjuvant. One week later, two subcutaneous injections in the neck region with a total of 50 μg of mBSA/ CFA were administered as a booster. Three weeks after the immunization, arthritis was induced in both knee joints by intra-articular injection of 60 μg of mBSA in 6 μl of saline.

Serum collection and antibody titer determination in serum

At day 7 and day 21 after AIA induction, blood was drawn from the retro-orbital plexus in MiniCollect tubes (Greiner Bio-One, Monroe, NC, USA), and subsequently serum was obtained by centrifugation. Anti-mBSA-specific antibodies (total IgG, IgG1, IgG2a, IgG2b) were measured in sera with an enzyme-linked immunosorbent assay. mBSA was coated on plates (Nunc; Thermo Fisher Scientific, Rochester, NY, USA) at a concentration of 100 μg/ml. Antibody concentrations were assessed by twofold serial dilution of the sera, followed by detection of bound mouse IgG with peroxidase-conjugated rabbit antimouse IgG (SouthernBiotech, Birmingham, AL, USA). 5-Aminosalicylic acid was used as a substrate. Absorbance was measured at 450 nm. Antibody titers were determined at 50% of the maximum absorption.

Lymphocyte stimulation test

Spleens were collected from mice at day 21 after AIA induction and homogenized through a cell strainer. Erythrocytes were lysed with lysis buffer (155 mM NH₄Cl, 12 mM KHCO₃, 0.1 mM ethylenediaminetetraacetic acid, pH 7.3). Cells were seeded into flasks, and after 1 hour at 37°C, nonadherent cells were harvested and seeded into 96 -well
plates (1x10^5 cells/well). mBSA was added at final concentrations of 50, 25, 12.5, 6.25, 3.12, and 1.56 μg/ml. Concanavalin A and ovalbumin were used as positive and negative controls, respectively. Cultures were maintained for 4 days. [3H]Thymidine was added for the last 16 hours of culture, and its incorporation was determined as a measure of T-cell proliferation.

**Histological analysis**

Total knee joints were isolated, fixed in 4% phosphate-buffered formalin, decalcified in 5% formic acid, embedded in paraffin, and 7-μm coronal sections of various depths of the joint were made. Sections were stained with H&E for histological analysis. Inflammation (infiltrate and exudate) was arbitrarily scored on a scale from 0 (no inflammation) to 3 (severe inflammation). Bone destruction was evaluated in 13 well-defined areas of the knee joint (as depicted in the scheme in Additional file 1a) with a score ranging from 0 (no erosion) to 3 (connection between joint cavity and bone marrow). For the evaluation of proteoglycan (PG) depletion as a measure of cartilage destruction, joint sections were stained with Safranin O and Fast Green. PG depletion was evaluated at both the patellofemoral and the tibiofemoral areas as the amount of red staining present, using an arbitrary score ranging from 0 (absence of PG depletion) to 3 (complete PG depletion). For quantification of the number of osteoclasts, total knee joint sections were stained for tartrate-resistant acid phosphatase (TRAP), using the Leukocyte Acid Phosphatase Kit (Sigma-Aldrich) according to the manufacturer’s protocol. The number of TRAP^+^ cells present along the external bone surface was counted. For quantification of periarticular bone, the percentage of noncartilage collagenous tissue (blue staining) in the complete femur and tibia of joint sections stained with Safranin O and Fast Green was quantified using Leica Application Suite software (Leica Microsystems, Buffalo Grove, IL, USA).

**Immunohistochemistry**

To visualize S100A8-, NIMPR14-, and F4/80 -expressing cells, knee joint sections were incubated with specific primary antibodies against S100A8 (made in our facilities), NIMPR14 (kindly provided by Dr. M. Strath, London, UK) and F4/80 (Thermo Fisher Scientific). Afterward, sections were incubated with horseradish peroxidase-conjugated or biotinylated secondary antibodies followed by avidin-biotin complex peroxidase (VECTORSTAIN Elite Kit; Vector Laboratories, Burlingame, CA, USA). Antibody binding was visualized using diaminobenzidine. S100A8 staining was arbitrarily scored using a scale from 0 to 3. For quantification of NIMPR14- and F4/80 -positive cells, pictures (original magnification ×100) of five specific areas of the joint were taken (two in the area adjacent to the patella and two in the area adjacent to the medial and lateral femur for
the evaluation of infiltrate, and one in the area of the joint cavity between the patella and femur for evaluation of the exudate). The amount of cells in the infiltrate was measured as the positive area above a fixed threshold using Leica Application Suite software (Leica Microsystems). The number of positive cells in the exudate was counted using the cell counter plugin of ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Flow cytometric analysis**

Bone marrow was isolated from femurs and tibias of mice by flushing the marrow cavity with medium and passing the cell suspension through a cell strainer. After lysis of erythrocytes, bone marrow cells were incubated with Fc -blocking antibody (BD Pharmingen antimouse CD16/CD32, clone 2.4G2; BD Biosciences, San Jose, CA, USA), followed by staining with the following mix of antibodies: CD11b-fluorescein isothiocyanate, CD90.2-phycoerythrin (PE), CD45R/B220-PE, CD49b-PE, NK1.1-PE, Ly6G-PE, Ly6C-allophycocyanin-cyanine 7 (all BD Biosciences). Samples were acquired with a CyAn flow cytometer (Beckman Coulter Life Sciences, Indianapolis, IN, USA), and data analysis was performed with Kaluza Analysis Software (Beckman Coulter Life Sciences). The gating strategy we used is depicted in Additional file 2.

**Bone marrow -derived osteoclast differentiation**

Bone marrow was isolated from femurs and tibias of mice. Total bone marrow cells were seeded into 96-well plates at a density of $10^5$ cells/well in 150 μl of α-minimum essential medium (Thermo Fisher Scientific), supplemented with 5% FCS, penicillin/streptomycin, 30 ng/ml recombinant mouse (rm)M-CSF, and 20 ng/ml rmRANKL (R&D Systems, Minneapolis, MN, USA). Culture medium was refreshed after 3 days.

**Measurement of TRAP activity in the supernatants**

Cell supernatants were collected after 5 days of differentiation, and TRAP activity was measured with a colorimetric assay. In short, p-nitrophenyl phosphate (New England Biolabs, Ipswich, MA, USA) was diluted in buffer containing 420 mM acetic acid (Sigma-Aldrich) and 160 mM tartrate solution (Merck, Kenilworth, NJ, USA) and added 1:1 to culture supernatant. After 1 hour, the reaction was stopped with 0.5 M NaOH (Sigma-Aldrich), and the absorbance at 405 nm was determined using a spectrophotometric plate reader (Bio-Rad Laboratories, Hercules, CA, USA).
**RNA isolation and qRT-PCR**

Well-defined synovial samples were isolated from the inflamed murine knee joints as previously described. Tissue samples were homogenized using the MagNA Lyser Instrument (Roche Diagnostics, Indianapolis, IN, USA). Total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). RNA from osteoclast cultures was isolated with TRIzol reagent (Sigma-Aldrich). RNA was subsequently reverse transcribed into complementary DNA. qRT-PCR was performed using the Applied Biosystems StepOnePlus RT-PCR System (Thermo Fisher Scientific, Foster City, CA, USA). Primer sequences are listed in Table 1 (primers obtained from Biolegio, Nijmegen, the Netherlands). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as the reference gene. Samples were normalized for the expression of Gapdh by calculating the comparative threshold: \( -\Delta Ct = - (Ct \text{ gene of interest} - Ct \text{ Gapdh}) \).

<table>
<thead>
<tr>
<th>Gene</th>
<th>PRIMER SEQUENCE (5′-3′)</th>
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| **Gapdh** | FW: GGCAAATTCAACGGCACA  
RV: GTTAGTGGGGTCTCGCTCCTG |
| **Nfatc1** | FW: ATGCGAGCCCATCATCGA  
RV: GGGATGTGAACCTCGGAAGAC |
| **Acp5** | FW: GACAAGAGGTTCAGGAGACC  
RV: GGGCTGGGGGAAGTCCAG |
| **Calcr** | FW: CGTTCTTTATTACCTGGCTCTTGTG  
RV: TCTGGCAGCTAAGGTTCTTGAA |
| **Mmp9** | FW: GGAACGCACGCATCCTTCACCA  
RV: GAAACTCACACGGAAGAAATTT |
| **Ca2** | FW: GCTGCAGAGGCTCAGTGGT  
RV: AAACAGCCAATCCATCCGGT |
| **Oscar** | FW: TGGTCATCGTTCGGAAGTTCTTCTG  
RV: CAGCCCCAAAACGGATGAG |
| **Dcstemp** | FW: TGTTATCGGCTCATCTCCTCAT  
RV: GACTCCCTGGGTCCCTGCTT |
| **Clcn7** | FW: AGCCCTGACTATGACACACCAG  
RV: GAAAGCCGTGTGGTGGTGGAT |
| **Ctsk** | FW: GAAGCAGTATAACAGCAGGGTGGAT  
RV: TGCTCCCAAAGTGGTGGATCGG |
Statistical analysis

Statistical differences between two groups were calculated using Student’s t-test for parametric variables (messenger RNA [mRNA] expression, IgG titer) or the Mann-Whitney U test for nonparametric variables (lymphocyte stimulation test/arbitrary score of inflammation, PG depletion, and S100A8 staining/percentage of osteoclast precursors/number of TRAP-positive cells in vivo/TRAP activity in the supernatant/quantification of Nimpr14- and F4/80-positive cells/percentage of non-cartilage calcified tissue). For comparison of multiple groups in the quantification of in vivo bone erosion and TRAP+ cells after in vitro differentiation, two-way analysis of variance was used. Spearman’s rank correlation coefficients \( r_S \) were calculated for correlation analysis. All analyses were performed using Prism version 5.03 software (GraphPad Software, La Jolla, CA, USA), and \( P \) values less than 0.05 were considered significant.

Results

Decreased bone erosion in FcγRI,II,III,IV-/- mice compared with WT controls

First, to determine the effect of the absence of FcγRs on bone resorption during experimental arthritis, we scored bone erosion after induction of AIA in FcγRI,II,III,IV-/- mice and their WT controls. At both day 7 and day 21 after AIA induction, we observed significantly decreased bone erosion in the FcγRI,II,III,IV-/- mice as compared with their WT controls, highlighting the importance of FcγRs in this process (Figure 1). However, although the bone erosion was significantly decreased in the FcγRI,II,III,IV-/- mice compared with their WT controls, scores were not reduced to the same level of in naive mice. To investigate a possible basal phenotype due to the absence of FcγRs, we determined the bone erosion in knee joints of WT and FcγRI,II,III,IV-/- mice without AIA. This showed comparable basal levels of bone erosion in these naive knee joints (Figure 1b). In addition, no differences were found in the surface area of noncartilage collagenous tissue present in the femur and tibia (Additional file 1b). Together, these findings suggest that the decreased bone resorption that was observed in the FcγRI,II,III,IV-/- mice after induction of experimental arthritis could not be explained by an underlying basal bone phenotype. As an additional readout of joint damage, we evaluated PG depletion as a measure of inflammation-induced cartilage destruction at the patellofemoral and tibiofemoral regions. In line with the decreased bone damage, we observed decreased PG depletion in FcγRI,II,III,IV-/- mice compared with their WT controls at day 7 and day 21 after AIA induction, particularly in the tibiofemoral area (Additional file 1c).
Figure 1. Fcγ receptor (FcγRI,II,III,IV−/−) mice have decreased bone erosion compared with wild-type (WT) control mice. 

a) Bone erosion (black arrows) is present in the joints of FcγRI,II,III,IV−/− and WT mice after induction of antigen-induced arthritis (AIA), as determined by using H&E-stained sections. Original magnification ×50 and ×200.

b) Quantification of bone erosion showed significantly decreased resorption in the joints of FcγRI,II,III,IV−/− mice compared with their WT controls (n = 17 and 24 joints per group respectively) at both 7 and 21 days after AIA induction. No basal differences in bone erosion could be observed in the joints of naive mice. Scatterplots are shown, with horizontal and vertical lines representing mean ± SEM values. ns Not significant. ** P < 0.01, *** P < 0.001 versus WT controls.
Comparable immune response against mBSA in WT and FcγRI,II,III,IV−/− mice

Because the induction of the AIA model is highly dependent on the immune response against the mBSA antigen, we set out to determine whether the humoral and cellular immune responses against mBSA were affected by the absence of FcγRI,II,III,IV. Therefore, we determined anti-mBSA IgG titers in the serum of mice with AIA. We found increased titers of total IgG, IgG1, and IgG2a, but a comparable titer of IgG2b, in FcγRI,II,III,IV−/− mice as compared with WT mice (Figure 2a). Moreover, using immunostaining against IgG in the joint, we showed IgG accumulation in both WT and FcγRI,II,III,IV−/− mice (Figure 2b). Finally, we observed comparable mBSA-induced proliferation of T cells obtained from FcγRI,II,III,IV−/− and WT mice (Figure 2c).

Absence of Fcγ receptors does not affect the number of osteoclast precursors

In the next set of experiments, we aimed at elucidating the mechanism underlying the decreased bone erosion observed in the absence of FcγRs. We first determined whether the FcγRI,II,III,IV−/− mice had a different percentage of osteoclast precursors in their bone marrow after induction of AIA. The percentage of CD11b^pos^ Ly6C^high^ and CD11b^low/neg^Ly6C^high^ cells, both of which have been shown to differentiate into osteoclasts 41, 42 were comparable between FcγRI,II,III,IV−/− and WT mice (Figure 3a). These data suggest that the observed decrease in bone erosion does not originate from differences in osteoclast precursor populations.

Absence of Fcγ receptors does not affect the osteoclastogenic potential and the number of osteoclasts on the bone surface during AIA

Next, we determined whether the osteoclast progenitors from FcγRI,II,III,IV−/− mice have the same osteoclastogenic potential as WT cells. After in vitro differentiation of bone marrow cells into osteoclasts with M-CSF and RANKL, we observed comparable numbers of multinucleated TRAP^+^ cells in the cultures with WT and FcγRI,II,III,IV−/− cells (Figure 3b). In agreement with this finding, we found comparable mRNA expression levels of key osteoclast differentiation markers, such as nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1), TRAP, dendritic cell-specific transmembrane protein (Dcstamp), calcitonin receptor (Ctr), and osteoclast-associated immunoglobulin-like receptor (Oscar), as well as comparable mRNA expression levels of activation markers, such as chloride channel 7 (Clcn7), carbonic anhydrase II (Ca2), matrix metallopeptidase 9 (Mmp9), and cathepsin K (Ctsk) (Figure 3c). Moreover, the TRAP enzyme activity as measured in the supernatant of the osteoclast cultures was comparable (Figure 3d). Together, these findings show that the absence of FcγRs does not affect the osteoclastogenic potential of precursor cells. Finally, because in vivo osteoclastogenesis is a complex process that can be influenced by many factors, we determined the number
of osteoclasts on the bone surface of FcγRI,II,III,IV⁻/⁻ and WT mice during AIA using TRAP staining. Interestingly, in line with their comparable osteoclastogenic potential in vitro, the number of TRAP⁺ cells along the bone surface did not differ between FcγRI,II,III,IV⁻/⁻ and WT mice both at day 7 and day 21 after AIA induction (Figure 3e).
Figure 3. Absence of Fcy receptor (FcyR) I, II, III, IV does not affect the percentage of osteoclast precursors and their osteoclastogenic potential. 

a. Comparable percentages of CD11b<sup>hi</sup>Ly6C<sup>hi</sup> and CD11b<sup>low</sup>/neg Ly6C<sup>hi</sup> osteoclast precursors were present in the bone marrow of wild-type (WT) and FcyRI,II,III,IV<sup>−/−</sup> mice at days 7 and 21 of antigen-induced arthritis (AIA) (n = 3 or 4 mice/group). 

b. Images of tartrate-resistant acid phosphatase (TRAP) staining of osteoclasts after 5 days of in vitro differentiation. Quantification showed comparable numbers of osteoclasts obtained from WT and FcyRI,II,III,IV<sup>−/−</sup> bone marrow cells (n = 5 mice/group). 

c. Moreover, comparable messenger RNA expression levels of various osteoclast markers were determined in WT and FcyRI,II,III,IV<sup>−/−</sup> osteoclasts (n = 3 mice/group). 

d. Finally, TRAP activity in the culture supernatant of WT and FcyR I,II,III,IV<sup>−/−</sup> macrophages and osteoclasts was comparable (n = 3 mice/group). 

Furthermore, comparable messenger RNA expression levels of various osteoclast markers were determined in WT and FcyRI,II,III,IV<sup>−/−</sup> osteoclasts (n = 3 mice/group). 

d. Finally, TRAP activity in the culture supernatant of WT and FcyR I,II,III,IV<sup>−/−</sup> macrophages and osteoclasts was comparable (n = 3 mice/group). 

e. Representative photomicrographs of TRAP staining and quantification of the number of positive cells along the bone surface in total knee joint sections of WT and FcyRI,II,III,IV<sup>−/−</sup> mice at days 7 and 21 after induction.
of AIA (n = 14 and n = 10 per time point for WT and FcγRI,II,III,IV⁻/⁻ mice, respectively). Scatterplots are shown, with horizontal and vertical lines representing mean ± SEM values. ns Not significant.

**FcγRs differentially regulate the influx of neutrophils present in the joint**

Because proinflammatory cells and their products can strongly increase the resorbing activity of osteoclasts, we evaluated the severity of inflammation in the arthritic knee joints. The degree of both infiltrate and exudate was significantly decreased in the knee joints of FcγRI,II,III,IV⁻/⁻ mice at day 7 after AIA induction (Figure 4). At day 21 after induction, the degree of inflammation was decreased in both strains, and no significant difference could be observed between FcγRI,II,III,IV⁻/⁻ and WT mice anymore. The early phase of inflammation (day 7) during AIA is particularly characterized by an abundant presence of neutrophils in the exudate and infiltrate in the knee joint. Interestingly, we observed significantly decreased numbers of NIMPR14 -positive neutrophils in the exudate, and the NIMPR14 -positive area in the infiltrate was significantly lower in FcγRI,II,III,IV⁻/⁻ mice than in WT mice (Figure 5a). In contrast, numbers of F4/80 -positive monocytes/macrophages in both the exudate and infiltrate were comparable between FcγRI,II,III,IV⁻/⁻ and WT mice (Figure 5b), and a trend toward an increased percentage of F4/80 cells in the infiltrate of FcγRI,II,III,IV⁻/⁻ mice was observed. Previous data developed at our laboratory showed that in contrast to the decreased inflammation and bone resorption in FcγRI,II,III,IV⁻/⁻ mice that we describe in the present work, FcγRI,II,III⁻/⁻ mice showed increased bone resorption, together with more inflammation, compared with their WT controls after induction of AIA. Of note, we observed a significant increase and a trend toward an increase in the numbers of neutrophils present in the infiltrate and exudate, respectively, of these FcγRI,II,III⁻/⁻ mice (Figure 5c). However, in agreement with our findings in the FcγRI,II,III,IV⁻/⁻ mice, the number of F4/80 -positive monocytes/macrophages cells was not significantly different between FcγRI,II,III⁻/⁻ and WT mice, and the percentage of F4/80 -positive area was decreased in FcγRI,II,III⁻/⁻ mice (Figure 5d). Representative photomicrographs are shown in Additional files 3 and 4. Together, these findings suggest that FcγRIV might be of particular importance in the recruitment of neutrophils into the arthritic joint and that these neutrophils likely contribute to the bone erosion process.
Figure 4. Fcγ receptor (FcγRI,II,III,IV<sup>-/-</sup>) mice have decreased inflammation in arthritic joints.
Photomicrographs of H&E staining showing the infiltrate and exudate in knee joints of wild-type (WT) and FcγRI,II,III,IV<sup>-/-</sup> mice at days 7 and 21 of antigen-induced arthritis (AIA). Original magnification ×100. Quantification showed decreased infiltrate and exudate in the knee joints of FcγRI,II,III,IV<sup>-/-</sup> mice compared with WT controls at day 7 of AIA. In contrast, no differences were observed at day 21 (n = 24 and n = 17 joints per time point for WT and FcγRI,II,III,IV<sup>-/-</sup> mice, respectively). Scatterplots are shown, with horizontal and vertical lines representing mean ± SEM values. ns Not significant. * P < 0.05, ** P < 0.01.

**Numbers of S100A8/A9-producing neutrophils strongly correlate with the amount of bone erosion**

In line with the decreased numbers of neutrophils present in their joints, lower expression levels of S100A8, at both the mRNA and protein levels, were observed in FcγRI,II,III,IV<sup>-/-</sup> than in WT mice (Figure 6a and b). Moreover, in agreement with the higher numbers of neutrophils observed in the joints of FcγRI,II,III<sup>-/-</sup> mice, increased expression of S100A8 was previously described by researchers at our laboratory. In support of our idea that the S100A8 produced by neutrophils plays an important role in the observed bone erosion, we found that the number of neutrophils in both the infiltrate and exudate, as well as their production of S100A8, strongly and significantly correlated with the severity of bone erosion (Figure 6c and d).
Figure 5. Prominent role for Fcγ receptor (FcγR)IV in regulating the influx of neutrophils into the joint.  

a) NIMPR14-positive area, percentage of NIMPR14-positive area in the infiltrate, and number of NIMPR14-positive cells in the exudate are decreased in FcγRI,II,III,IV−/− mice compared with wild-type (WT) mice in day 7 antigen-induced arthritis (AIA). 

b) Comparable numbers of F4/80-positive cells are present in the joints of FcγRI,II,III,IV−/− mice compared with their WT controls at day 7 AIA. 

c) In contrast to FcγRI,II,III,IV−/− mice, the total NIMPR14-positive area, percentage of NIMPR14-positive area in the infiltrate, and number of NIMPR14-positive cells in the exudate are increased in FcγRI,II,III−/− mice. 

d) Comparable numbers and reduced percentage of F4/80-positive cells in the joint of FcγRI,II,III−/− mice compared with their WT controls at day 7 of AIA. Scatterplots are shown, with horizontal and vertical lines representing mean ± SEM values of at least six mice per group. ns Not significant. * P < 0.05, ** P < 0.01
Figure 6. The number of S100A8 producing neutrophils correlates with the severity of bone erosion during antigen-induced arthritis.  

**a** S100a8 messenger RNA levels in the synovium of Fcγ receptor (FcγRI,II,III,IV−/−) mice are decreased compared with wild-type (WT) mice at day 7 of antigen-induced arthritis (AIA).  

**b** Representative photomicrographs show S100A8 staining in knee joint sections of WT and FcγRI,II,III,IV−/− mice at day 7 of AIA. Original magnification ×100 and ×400. Quantification showed a decreased number of S100A8-positive cells in FcγRI,II,III,IV−/− mice compared with their WT controls.  

**c** A significant correlation was observed between the number of neutrophils (NIMPR14-positive cells) in the infiltrate and exudate in the joint and bone erosion in both WT and FcγRI,II,III,IV−/− mice at day 7 of AIA.  

**d** A significant correlation between the number of S100A8-positive cells in the joint and the severity of bone erosion in both WT and FcγRI,II,III,IV−/− mice at day 7 of AIA was found. *P < 0.05.*  

rs = Spearman’s rank correlation coefficient.
Discussion

In the present study, we show that FcγRs are crucially involved in bone erosion during AIA. Moreover, we show that the absence of all FcγRs does not affect the number of osteoclast precursors or their osteoclastogenic potential, but that it decreases the subsequent bone erosion during experimental arthritis via a reduction of inflammation. The comparison of development of AIA in FcγRI,II,III,IV<sup>-/-</sup> and FcγRI,II,III<sup>-/-</sup> mice suggested a possible crucial role of FcγRIV in mediating neutrophil inflammation during AIA.

We observed that mice lacking all FcγRs had decreased inflammation at day 7 and decreased bone erosion at both day 7 and day 21 after induction of the disease. This is in agreement with a previous study by Hobday and colleagues, which showed that FcγRI,II,III,IV<sup>-/-</sup> mice were completely protected from serum-transferred arthritis at a macroscopic level. However, in contrast to this serum transfer model, the AIA model is characterized by clear T-cell involvement, which is probably FcγR-independent. This might explain why we did not observe complete protection in FcRI,II,III,IV<sup>-/-</sup> mice. In another study, in line with what we observed in FcγRI,II,III,IV<sup>-/-</sup> mice, y-chain/FcγRIIb<sup>-/-</sup> mice lacking signaling of both activating and inhibitory FcγRs, as well as of other receptors using the y-chain, were completely protected from development of CIA, although bone erosion data were not reported.

The induction of the AIA experimental RA model is highly dependent on the binding of mBSA/anti-mBSA-containing ICs to FcγRs, thereby potently activating the cell, and on the activation of T-cell responses against mBSA. However, we observed comparable T-cell responses against the mBSA antigen in FcγRI,II,III,IV<sup>-/-</sup> and WT animals, which shows that their contribution to the induction of the model was not affected by the absence of the FcγRs. This is consistent with previous studies in which normal T-cell responses were found in the absence of either activating or inhibitory FcγRs after the induction of the AIA. Together, these findings suggest that the development of the T-cell immune response is FcγR-independent.

It has previously been shown that the absence of FcγRIIb often leads to increased IgG titers in mice owing to a lack of negative feedback on the production of IgGs by plasma cells, which results in enhanced stimulation of the immune response. In line with these findings, the FcγRI,II,III,IV<sup>-/-</sup> mice used in this study, which also lack FcγRIIb, showed increased IgG titers (total IgG, IgG1, IgG2a) compared with their WT controls. Moreover, accumulation of IgG was present in the joints of FcγRI,II,III,IV<sup>-/-</sup> mice. Together, these data showing a normal T-cell response against mBSA and increased IgG titers in FcγRI,II,III,IV<sup>-/-</sup> mice suggest that the observed decrease in bone pathology cannot be the result of an insufficient immune response against mBSA after induction of the AIA model.
Osteoclasts are the main cells responsible for the degradation of bone tissue during RA. Although it is known that osteoclasts differentiate from bone marrow-derived myeloid precursors under the influence of M-CSF and RANKL, a strictly defined osteoclast precursor set has not yet been identified. CD11b\textsuperscript{pos} Ly6C\textsuperscript{high} bone marrow monocytes have been reported to differentiate into osteoclasts when stimulated with M-CSF and RANKL in vitro, and depletion of Ly6C\textsuperscript{high} cells in vivo results in decreased osteoclast formation and subsequent bone resorption in the K/BxN serum transfer model. Moreover, Charles and colleagues recently identified the CD11b\textsuperscript{low/neg} Ly6C\textsuperscript{hi} BM population as having osteoclastogenic potential both in vitro and in vivo. In the present study, we did not observe differences in the relative percentages of both the Ly6C\textsuperscript{high} and CD11b\textsuperscript{low/neg} Ly6C\textsuperscript{high} osteoclast precursor populations between FcγRI,II,III,IV\textsuperscript{-/-} and WT mice, which allowed us to exclude the possibility that decreased bone erosion in the FcγRI,II,III,IV\textsuperscript{-/-} mice was merely the result of decreased numbers of osteoclast precursors.

Next to M-CSF and RANKL signaling, a costimulatory signal via the activation of the ITAM domain, which is present in the γ-chain and in DNAX activation protein of 12 kDa (DAP-12), is required for the activation of NFATc1, which is the transcription factor essential for osteoclast differentiation. Because activating FcγRI, FcγRIII, and FcγRIV are expressed by osteoclasts and are associated with the ITAM-containing γ-chain, they potentially can affect osteoclast differentiation. However, in the present study, we found that the absence of all four FcγRs does not impact the differentiation of osteoclasts from their precursors in vitro. Underlining our findings, Negishi-Koga and colleagues showed that FcγRIIb\textsuperscript{-/-}/γ-chain\textsuperscript{-/-} cells, which lack signaling of both activating and inhibitory FcγRs, did not show alterations in osteoclast differentiation. However, varying results have previously been described concerning the effects of FcγRs on osteoclastogenesis. Surprisingly, Negishi-Koga and colleagues reported that FcγRIII\textsuperscript{-/-} mice have an osteoporotic phenotype that was associated with increased numbers of osteoclasts. These authors also demonstrated increased in vitro osteoclast differentiation of FcγRIII\textsuperscript{-/-} bone marrow cells, together suggesting an inhibitory role for FcγRIII in osteoclastogenesis. They stated that the mechanistic basis for this surprising finding could be the sequestration of the γ-chain by FcγRIII. Therefore, in the absence of FcγRIII, more γ-chain is available for other proteins, such as osteoclast-associated immunoglobulin-like receptor (OSCAR) and paired immunoglobulin-like receptor A (PIR-A), which both act as costimulatory factors during osteoclastogenesis via their association with the γ-chain. In addition, the same research group showed that FcγRIIb\textsuperscript{-/-} cells, which lack the ITIM domain that inhibits pro-osteoclastogenic ITAM signaling, showed increased osteoclastogenic potential. Therefore, in the present study, where we show that FcγRI,II,III,IV\textsuperscript{-/-} cells have normal in vitro osteoclastogenic potential, we cannot rule out a compensatory effect on osteoclast differentiation via γ-chain-dependent
costimulatory pathways, such as via OSCAR or PIR-A signaling, or via triggering receptor expressed on myeloid cells 2 (TREM2), which depends on the ITAM-domain containing DAP12 protein for its signaling. In line with this possibility, it has been shown that DAP12 is primarily responsible for *in vitro* M-CSF- and RANKL -induced osteoclastogenesis because γ-chain/- cells show normal differentiation. However, in the absence of DAP12, the γ-chain can compensate for its absence, acting via αvβ3 integrin.

Finally, underlining the normal osteoclast differentiation that we observed *in vitro*, nonarthritic FcγRI,II,III,IV/- mice had no basal bone phenotype in their knee joints, measured as the amount of subchondral calcified tissue in the femur and tibia, and we observed comparable numbers of TRAP+ osteoclasts along the bone surface after AIA induction. Together, these findings imply that the absence of all FcγRs does not impair the ability of cells to differentiate into mature osteoclasts, suggesting that a difference in osteoclast activity, rather than differences in osteoclast numbers, must underlie the decreased erosion in FcγRI,II,III,IV-/- mice in the studied AIA model.

It has been shown that inflammation plays a critical role in the activation of osteoclasts *in vivo* by the production of a plethora of factors that in this way lead to bone erosion. In this study, we found that inflammation (both infiltrate and exudate) was decreased in FcγRI,II,III,IV/- mice compared with WT controls at day 7, but not at day 21, after AIA induction. This suggests that FcγRs are probably most involved in the early inflammatory response of the AIA model, which still resulted in significantly decreased bone erosion in the FcγRI,II,III,IV-/- mice at day 21 after induction.

It has been shown that the four FcγRs in mice are differentially expressed on immune cells and that the individual FcγRs are known to bind the various IgG subclasses with different affinities. The activity of IgG1 is dependent mainly on FcγRIII, and IgG2a binds with high affinity to FcγRI and with low affinity to FcγRIII and IV, whereas IgG2b bind with the highest affinity to FcγRIV. This implies that the absence of one or more FcγRs and the cellular composition might facilitate the binding of IgGs to other FcγRs more than normally would occur, possibly leading to abnormal intracellular signaling. Previous researchers have investigated the role of FcγRs in AIA using mice deficient in one or more FcγRs. Induction of AIA in FcγRIIb/- mice resulted in increased inflammation, most probably because of the absence of both FcγRIIb-mediated IgG clearance and a negative feedback loop on IgG production by B cells. In contrast, the inflammation in FcγRI/- or FcγRIII/- mice was not affected, suggesting that either these receptors can compensate for each other’s absence or that FcγRIV is the dominant FcγR in both cases. However, the combined absence of FcγRI and FcγRIII led to reduced inflammation. Interestingly, when combined with the absence of the IgG -clearing FcγRIIb, resulting in FcγRI,II,III/- mice, stronger accumulation of ICs is present, which results in increased inflammation, probably via binding of ICs to FcγRIV.
in this situation is substantiated by the finding that the inflammation is reduced when, in addition to FcγRI, FcγRII, and FcγRIII, FcγRIV is also absent. Although our results indicate an important role for FcγRIV in the pathology of AIA, definitive proof should come from AIA induction in an FcγRIV single-knockout. Consistent with our findings in the AIA model, the fact that 35–40% of FcγRI,II,III/- mice developed CIA, whereas γ-chain-/-/FcγRIIb-/- mice that also lack functional FcγRIV were protected from disease development, further supports the role of FcγRIV in this arthritis model. Moreover, a clear confirmation for the crucial involvement of FcγRIV in K/B×N comes from a study by Seeling and colleagues in which FcγRIV-/- animals showed significantly decreased inflammation and bone erosion. Finally, arthritis development can be prevented using a blocking antibody against FcγRIV in mice lacking all FcγRs except for FcγRIV.

Together, these studies and the data reported in the present article show that an important role can likely be attributed to FcγRIV in experimental RA models. Interestingly, a polymorphism in FcγRIIIA, which is the human ortholog of the murine FcγRIV, has been associated with increased susceptibility to RA development. This polymorphism, in which a phenylalanine is substituted for a valine at amino acid position 158 (158 V/F), located at the immunoglobulin-binding domain, results in an increased affinity of the receptor for IgG1 and IgG3 antibodies. Although these associations could not be reproduced in all populations, these data support a possible important role for the human ortholog of murine FcγRIV in RA development and further support the importance of additional studies to investigate the role of FcγRs in RA.

In our present study, we describe a clear increase in the numbers of neutrophils in both the infiltrate and exudate in FcγRI,II,III/- mice, whereas decreased neutrophil numbers were observed in FcγRI,II,III,IV/- mice. This suggests that particularly FcγRIV is responsible for the presence of neutrophils to the joint. The importance of neutrophils in RA development has been shown in the K/B×N experimental RA model. Moreover, in agreement with this role for neutrophils in experimental RA models, high numbers of neutrophils are present in the joints of patients with active RA. We show a strong and significant correlation between the number of neutrophils present in the joint and the amount of bone erosion. Because in the AIA model ICs are produced locally in the joint, we propose that ICs can stimulate synovial macrophages via FcγRIV to release chemotactic factors such as complement components and keratinocyte-derived chemokine, thereby inducing the recruitment of neutrophils into the joint. Neutrophils are strong producers of the alarmin S100A8/A9, which has been shown to directly induce osteoclast activity in vitro via TLR4 signaling. This suggests a mechanism through which infiltrated neutrophils can regulate bone erosion in RA. Indeed, we observed lower expression levels of S100A8 coinciding with lower neutrophil numbers in the joints of FcγRI,II,III,IV/- mice than in WT mice. Moreover, we observed that the
production of S100A8 by neutrophils strongly and significantly correlated with the severity of bone erosion. In agreement with these findings, our laboratory previously showed that FcγRI,II,III−/− mice, which have increased numbers of neutrophils in their joints after AIA induction, showed increased S100A8 expression. S100A8/A9 has been shown to be strongly upregulated in the synovial fluid of patients with RA37, 38, and its levels are linked to joint inflammation and damage 55-58. Together, these data support the idea that FcγRIV mediates bone erosion in AIA by modulating the influx of S100A8/A9-producing neutrophils into the arthritic joint, although an additional direct effect of ICs on osteoclasts cannot be completely ruled out.

**Conclusions**

Our present study adds important new data to the existing body of knowledge concerning the involvement of FcγRs in inducing bone erosion and particularly highlights, for the first time to our knowledge, the role of FcγRIV in neutrophil-mediated bone erosion during AIA.

**Acknowledgements**

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CHAPTER 3

References

Graphical representation of bone erosion scoring method, quantification of non-cartilage collagenous tissue and proteoglycan depletion.

**A)** Graphical representation of the 13 locations along the patella, femur, tibia and cruciate ligament where bone erosion was scored. **B)** Quantification of the non-cartilage collagenous tissue (blue staining in Safranin O/Fast Green staining) in femur and tibia showed no differences between naive FcγRI,II,III,IV−/− and wild type mice. *ns*=not significant. **C)** Quantification of PG depletion showed a significant decrease at the tibio-femoral area in the joints of FcγRI,II,III,IV−/− mice as compared to their WT controls (n=10 and 14 joints per group respectively) at both 7 and 21 days after AIA induction. Scatter plots are shown, with horizontal and vertical lines showing mean +/- SEM values. *ns*=not significant; * = P < 0.05 ** = P < 0.01; versus WT controls.
Gating strategy for flow cytometry analysis. Gating strategy for flow cytometry analysis used to identify CD11b\textsuperscript{pos} Ly6C\textsuperscript{high} and CD11b\textsuperscript{low/neg} Ly6C\textsuperscript{high} osteoclast precursor populations. First, single cells were selected. For identification of CD11b\textsuperscript{pos} Ly6C\textsuperscript{high} monocytes, cells negative for CD90.2, CD45R/B220, CD49b, NK1.1, Ly6G and positive for CD11b were selected (gate A). Subsequently, cells were back gated for side scatter and FS to exclude cells with high granulosity (gate B) and finally Ly6C\textsuperscript{high} cells were selected (gate C). For identification of CD11b\textsuperscript{low/neg} Ly6C\textsuperscript{high}, after exclusion of CD90.2, CD45R/B220, CD49b, NK1.1, Ly6G positive cells (gate D), cells were gated for their expression of CD11b and Ly6C (CD11b\textsuperscript{low/neg} Ly6C\textsuperscript{high}) (gate E).
**NIMPR14 and F4/80 positive cells in the infiltrate and in the exudate in the joints of FcγRI,II,III,IV−/− mice and their WT controls.**

**A)** Representative photomicrographs of NIMPR14 and **B)** F4/80 stainings showing neutrophils and macrophages in the infiltrate and in the exudate of the knee joints of FcγRI,II,III,IV−/− mice and their WT controls at day 7 after induction of antigen-induced arthritis. Original magnification 400X for infiltrate and 200x and 400x for exudate.
NIMPR14 and F4/80 positive cells in the infiltrate and in the exudate in the joints of FcyRI,II,III−/− mice and their WT controls.

A) Representative photomicrographs of NIMPR14 and B) F4/80 stainings showing neutrophils and macrophages in the infiltrate and in the exudate of the knee joints of FcyRI,II,III−/− mice and their WT controls at day 7 after induction of antigen-induced arthritis. Original magnification 400X for infiltrate and 200x and 400x for exudate.
High LDL-C levels attenuate onset of inflammation and cartilage destruction in antigen-induced arthritis

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Abstract

Background: In this study, we used hypercholesterolaemic apolipoprotein E-deficient (Apoe−/−) mice to investigate LDL/oxLDL effect on synovial inflammation and cartilage destruction during antigen-induced arthritis (AIA). Further, as macrophage FcγRs are crucial to immune complex-mediated AIA, we investigated in vitro the effects of high cholesterol levels on the expression of FcγRs on macrophages.

Methods: AIA was induced by intra-articular injection of mBSA into knee joints of immunized Apoe−/− and wild type (WT) control mice. Joint swelling was measured by uptake of 99mTc pertechnetate (99mTc). Joint inflammation and cartilage destruction were assessed by histology. Anti-mBSA IgGs were measured by ELISA and specific T-cell response by lymphocyte stimulation test. Upon oxLDL stimulation of WT macrophages, protein levels of FcγRs were measured by flow cytometry.

Results: Local induction of AIA resulted in less joint swelling, synovial infiltrate and exudate in the joint cavity in Apoe−/− mice compared to WT controls, even though both their humoral and adaptive immune response were comparable. Whereas Apoe deficiency alone did not affect macrophage expression of FcγRs, oxLDL sharply reduced the protein levels of activating FcγRs, crucial in mediating cartilage damage. In agreement with the reduced inflammation in Apoe−/− mice, we observed decreased MMP activity and destruction in the articular cartilage.

Conclusion: Taken together, our findings suggest that high levels of LDL/oxLDL during inflammation, dampen the initiation and chronicity of joint inflammation and cartilage destruction in AIA by regulating macrophage FcγR expression.
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects 1-2% of the world population and is characterized by severe joint inflammation and destruction. RA is largely driven by intra-articular immune complexes (ICs), consisting of IgG class immunoglobulins together with their cognate antigen, which predominantly interact with macrophages via binding to Fcγ receptors (FcγRs). Previous studies have shown that synovial macrophages are crucial in the onset and propagation of experimental IC-mediated arthritis. In mice, macrophages express four subtypes of FcγRs (FcγRI, FcγRIIb, FcγRIII, FcγRIV), which differ in their affinity for the various IgG isotypes as well as in their function. FcγRI, FcγRIII and FcγRIV induce cell activation upon binding with ICs, while FcγRIIb inhibits cell activation and is involved in IC removal. Skewing of the FcγR balance on the surface of synovial macrophages towards activating FcγRs strongly increases the severity of joint inflammation and destruction.

We showed that FcγRI plays a predominant role in mediating cartilage destruction and chondrocyte death via activation of matrix metalloproteases (MMPs). On the other hand, mice lacking FcγRIIb showed increased inflammation and cartilage destruction, thus confirming the inhibiting effects of FcγRIIb. Moreover, mice lacking FcγRI, Iib and III showed increased IC retention and inflammation, suggesting an additional pathological role for FcγRIV in this experimental model. RA is often associated with atherosclerosis, characterized by high levels of low-density lipoprotein cholesterol (LDL-C). Early RA is marked by increased serum levels of total cholesterol (TC) and LDL-C, in contrast, active RA and chronic inflammation are accompanied by a reduction of TC and LDL-C serum levels, although the clinical effect of high LDL-C levels in the initiation and progression of RA is still a matter of debate. Hence, we set out to determine how high systemic LDL-C levels modulate the onset and progression of disease. During inflammation, LDL-C levels in the joint resemble those in the blood, implying that people with high circulating LDL-C levels are subjected to have higher levels of LDL-C present in their joints. In an inflamed joint, IC-mediated cell activation leads to the production of reactive oxygen species (ROS) and results in the oxidation of LDL-C, thereby forming oxLDL, which is taken up by macrophages via scavenger receptors CD36, SR-A and LOX-1 leading to cell activation. Previous studies have shown contradictory results of high cholesterol levels on the development of arthritis. However, the effects of high cholesterol levels on the expression of the immune-regulating FcγRs and their interplay with ICs remains to be elucidated. Therefore, in the present study we investigated the effects of high LDL-C levels on the development of cartilage destruction during experimental RA by induction of the IC-driven antigen-induced arthritis (AIA) model in Apolipoprotein E deficient (Apoe-/-) mice, which spontaneously develop high LDL-C levels, and their wild type (WT) controls.
Furthermore, as FcγRs are crucial to IC-mediated stimulation, we deepened our understanding of the effects of high cholesterol levels on the expression of FcγRs on macrophages.

**Methods**

**Animals**

Apoεε<sup>−/−</sup> mice (JAX strain) were obtained from the Charles River Laboratory. Wild type C57Bl/6J mice were used as controls. All mice (10 mice/group) were housed in filter-top cages and received a standard chow diet and acidified water *ad libitum*. Male mice between 10-12 weeks were used in all the experiments, which were performed in accordance with the Dutch regulations and guidelines for care and use of laboratory animals. All animal studies were approved by the Radboud University’s Animal Experiment Committee, Nijmegen-the Netherlands (RU-DEC 2014-191).

**Induction of antigen–induced arthritis**

Mice were immunized with 100 µg/mL of methylated bovine serum albumin (mBSA; Sigma–Aldrich) as previously described<sup>8</sup>. Three weeks after immunization, arthritis was locally induced by intra-articular injection of 60 µg of mBSA in 6 µl saline in the knee joint.

**99mTc pertechnetate uptake measurement**

Joint swelling was measured by 99mTc pertechnetate uptake (<sup>99m</sup>Tc) in the knee joint and scored as previously described<sup>23</sup>.

**Histology**

Total knee joints were dissected, fixed in phosphate-buffered formalin (pH 7.4), decalcified in ethylenediaminetetraacetic acid (EDTA) and subsequently embedded in paraffin. Coronal sections of 7 µm representing the whole joint were stained with hematoxylin & eosin (H&E) or Safranin O (SafO). See **Supplementary material** for the scoring of histological parameters.

**Determination of cholesterol levels and IgG titres in serum**

Total cholesterol, LDL-C and high-density lipoprotein cholesterol (HDL-C) levels were determined in the serum prior to induction of AIA in naive Apoeεε<sup>−/−</sup> mice and WT controls (3 mice/group), and at day 21 after AIA (10 mice/group). Values were calculated based on the Friedewald formula<sup>24</sup>. The production of anti-mBSA specific antibodies (total IgG,
IgG1, IgG2a and IgG2b) was determined by enzyme-linked immunosorbent assay (ELISA) as previously described 11.

**Lymphocyte stimulation test**

Spleens were collected (4 mice/group) at day 21 after AIA induction and homogenized through a cell strainer. Erythrocytes were lysed with lysis buffer (155 mM NH₄Cl, 12 mM KHCO₃, 0.1 mM ethylenediaminetetraacetic acid, pH 7.3). Cells were seeded into flasks and after 1h at 37 °C non-adherent cells were harvested and seeded into 96-well plates (1 x 10⁵ cells/well). Cultures were maintained for 4 days in presence of 2-fold serial dilution of mBSA (starting with 25µg/mL) and for the last 16 hours ³H-Thymidine was added. Its incorporation was determined as a measure of T-cell proliferation.

**Luminex**

Levels of cytokines and chemokines were measured in serum samples using Luminex multyanalyte technology and multiplex cytokine kits (Milliplex; Millipore), which sensitivity was < 1pg/mL.

**Culture of macrophages**

Macrophages were differentiated from bone marrow-derived cells (BMDCs), previously isolated from naive WT and Apoe⁻/⁻ mice by flushing the marrow cavity with DMEM using a syringe. BMDCs were differentiated into macrophages by culturing them for 6 days in Dulbecco’s Modified Eagle's medium (DMEM), supplemented with 15 ng/mL recombinant mouse macrophage-colony stimulating factor (RmM-CSF, R&D Systems), 10% fetal calf serum (FCS, Thermo Scientific), 1mM pyruvate and 1% penicillin and streptomycin (P/S).

**oxLDL preparation and stimulation of macrophages**

LDL was isolated by single-spin density gradient ultracentrifugation from EDTA-treated blood from healthy volunteers and oxidised as previously described 25. Bone marrow-derived macrophages (BMDMs) were then stimulated with either 10 µg/mL oxLDL or LDL-C as control. This concentration was chosen to resemble the LDL-C levels in vivo 26.

**Flow cytometry**

BMDMs stimulated for 24h with LDL-C or oxLDL and their unstimulated controls were washed with PBS and scraped from 24-well plates by using 10mM EDTA/PBS. See **Supplementary materials** for the antibodies used to determine FcγR expression.
**Immunohistochemistry**

Immunostaining was performed on whole joint sections to detect the presence of the neo-epitope VDIPEN, which is exposed following aggrecan cleavage by matrix metalloproteases (MMPs). A specific antibody directed against the VDIPEN cleaved site was used (2.5 µg/mL) and Rabbit IgG was used as isotype control. The amount of staining present in the patellofemoral and tibiofemoral areas was quantified in three consecutive sections per knee joint using an arbitrary score on a scale from 0 to 3.

**Statistics**

Statistics were performed using Graph Pad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). Differences between the two groups were tested using a two-tailed Student’s t-test for comparing parametric variables, Mann-Whitney test for non-parametric variables (e.g. histological score) and multiple comparisons were tested using One–way ANOVA followed by Bonferroni’s multiple comparison test. \( P \) values <0.05 were considered significant. Data are presented as the mean ±95% CI.

**Results**

*Apoe\(^{-/-}\) mice develop less inflammation after induction of AIA*

First, we determined the effects of high cholesterol levels on joint inflammation after induction of AIA in knee joints of previously immunised *Apoe\(^{-/-}\)* mice and their WT controls. As expected, serum levels of total cholesterol (TC) in *Apoe\(^{-/-}\)* mice were significantly higher than in WT controls, mainly due to a sharp increase of LDL-C levels. Of note, the induction of AIA in *Apoe\(^{-/-}\)* mice reduced the serum levels of TC, as result of decreased LDL-C and HDL-C compared to naive mice (Figure 1A). *Apoe\(^{-/-}\)* mice showed significantly decreased \(^99\)Tc uptake as readout for joint swelling at days 1, 3, and 7 after induction of AIA as compared to WT controls (a reduction of 21%, 17% and 18%, respectively). However, at day 14 after induction, joint swelling was strongly reduced in both strains and the difference was lost (Figure 1B). Underlining a difference in the early inflammatory response, in histology we observed a significant reduction in both the infiltrate and exudate in the knee joints of *Apoe\(^{-/-}\)* mice (22% and 44% lower, respectively) at day 21 after induction (Figure 1C and D). To determine whether *Apoe\(^{-/-}\)* mice had a basal difference in synovial cellularity, we additionally scored contralateral control joints. However, no differences were found in infiltrate (Figure 1E), whereas exudate was absent in these naive knee joints.
Figure 1. Apoe<sup>-/-</sup> mice develop less joint inflammation during AIA. A) The levels of total cholesterol (TC), LDL-C and HDL-C were determined in the serum of WT and Apoe<sup>-/-</sup> naïve and arthritic mice at day 21 AIA. Naïve Apoe<sup>-/-</sup> mice show significantly higher levels of TC than WT controls, mainly due to higher LDL-C. Notably, after AIA induction TC and LDL-C levels decreased, however yet they were 3.8 times higher in Apoe<sup>-/-</sup> mice when compared to WT controls. Such increase was determined by higher LDL-C levels rather than HDL-C (55 vs. 1.6 fold higher, respectively). Horizontal and vertical lines represent the mean ±95% CI of 3 mice (naïve) or 10 mice (arthritic). B) R/L ratios of $^{99m}$Tc uptake at day 1, 3, 7 and 14 after intra-articular injection of mBSA into the knee joints of mBSA–immunized Apoe<sup>-/-</sup> mice and their wild type (WT) controls. Note that WT mice display significantly higher joint swelling compared to Apoe<sup>-/-</sup> mice. C) Representative images of cell infiltrate and exudate as determined by histology in Apoe<sup>-/-</sup> mice and WT controls at day 21. D) Quantification of cell infiltrate and exudate showed that arthritic Apoe<sup>-/-</sup> mice had a significant reduction of infiltrate and exudate as compared to WT controls. E) However, contralateral knee joints of WT and Apoe<sup>-/-</sup> mice had no signs of cell infiltration. Horizontal and vertical lines represent the mean ±95% CI of 8 mice (contralateral joints) or 10 mice (arthritic). (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, ns = not significant). Original magnification, 100 x.
The immune response is comparable in arthritic wild type and Apoe<sup>−/−</sup> mice

Because the induction of the AIA model is highly dependent on the formation of ICs that can bind to FcγRs, we determined whether Apoe deficiency influenced serum IgG titres. This would affect the amount and isotypic composition of ICs present in the joint, resulting in a less robust stimulation of FcγRs. However, we found that levels of total IgG, IgG1, IgG2a and IgG2b against mBSA in the serum were not significantly different between Apoe<sup>−/−</sup> and their WT controls at day 21 of AIA (Figure 2A). Further, we determined the T-cell response against mBSA and found no significant differences (Figure 2B), suggesting that the reduced inflammation observed in Apoe<sup>−/−</sup> mice is not due to an impaired humoral or adaptive immune response.

**Figure 2.** The immune response is comparable between arthritic WT and Apoe<sup>−/−</sup> mice. A) No significant differences were found in the production of anti-mBSA antibodies (total IgG, IgG1, IgG2a and IgG2b) between Apoe<sup>−/−</sup> mice and their WT controls (n = 10 mice/group). Mean represents the two-log values using 50% of the maximal extinction as an endpoint. B) The cellular immune response to mBSA, as determined by T-cell proliferation, was comparable between WT and Apoe<sup>−/−</sup> mice. Results are expressed as stimulation index (ratio of stimulation with/without antigen) (n = 4 mice/group). Horizontal and vertical lines represent the mean ±95% CI.
**Apoe deficiency does not affect basal levels of FcγRs on macrophages**

Since we observed no differences in the immune response or in the systemic production of pro-inflammatory cytokines (Figure S1) that could explain the reduced inflammation, we next determined whether Apoe deficiency affected the expression of the receptors for ICs, the FcγRs, which are crucial in regulating the acute phase of AIA. Since macrophages play a pivotal role in the onset and propagation of disease, we compared the expression of FcγRs in Apoe⁻/⁻ and WT BMDMs. However, the protein levels of FcγRs on the cell surface of Apoe⁻/⁻ and WT BMDMs were not different as indicated by comparable MFI levels (FcγRI 44.2 vs 44.6; FcγRIIb 43.9 vs 43.8; FcγRIII 11.2 vs 11.3; FcγRIV 32.1 vs 32, respectively), suggesting that APOE is not involved in regulating basal FcγR expression (Figure 3A and 3B).

![Figure 3](image-url)  
**Figure 3. Apoe deficiency does not affect FcγR levels on macrophages.** A) Histograms of WT and Apoe⁻/⁻ BM-derived macrophages (BMDMs) showing the expression of the various Fcγ receptors (FcγRs). B) Mean fluorescence intensity (MFI) show that WT and Apoe⁻/⁻ BMDMs express comparable levels of FcγRs. One experiment representative of two independent experiments is shown.

**oxLDL down-regulates the levels of FcγRI, II and IV on macrophages**

APOE is important in lipid transportation and its absence spontaneously leads to high systemic levels of LDL-C, which is oxidised into oxLDL in an inflammatory milieu. As FcγR levels were similar in WT and Apoe⁻/⁻ BMDMs, we next determined whether high LDL-C and oxLDL levels altered the expression of FcγRs. We stimulated WT BMDMs with either LDL-C or oxLDL for 24 hours *in vitro*, determined their lipid uptake by Oil Red O staining (Figure 4A) and performed flow cytometry to assess the protein levels of FcγRs on the
cell membrane. In contrast to LDL-C, oxLDL accumulated within the cells and strongly lowered the expression of FcγRI and FcγIV (38% and 20%, respectively), while the expression of FcγRIII was not changed (Figure 4B and 4C). Further, we found that both LDL-C and oxLDL, mildly reduced the expression of inhibiting FcγRIIb (10% and 16% lower, respectively). This dataset indicates that oxLDL leads to a strong reduction in the expression of activating FcγRI and IV on macrophages, thus underlining a role of oxLDL in modulating the innate immune response.

Figure 4. oxLDL down-regulates the levels of FcγRs on macrophages. WT bone marrow-derived macrophages (BMDMs) were cultured for 24 hours with or without 10µg/mL LDL-C or oxLDL. A) Oil Red O staining was used to investigate the uptake of LDL-C and oxLDL, which in contrast to LDL-C accumulated within the cell (black arrows). Original magnification, 100x. B) Histograms of non-stimulated (NS), LDL-C or oxLDL-stimulated BMDMs are shown. C) Mean fluorescence intensity (MFI) of FcγR expression in NS, LDL-C or oxLDL-stimulated BMDMs are shown. Both LDL-C and oxLDL significantly reduced the levels of FcγRIIb, while only oxLDL strongly reduced the activating FcγRI and IV (38% and 20% reduction, respectively). Conversely, the expression of FcγRIII remained unchanged. Horizontal and vertical lines represent the mean ±95% CI of one experiment representative of two independent experiments. * = P < 0.05, ** = P < 0.01, *** = P < 0.001 vs. the NS control; # = P < 0.05 ## = P < 0.01, ### = P < 0.001 vs. LDL-C stimulation.
**Figure 5.** *Apoe*⁻/⁻* mice show reduced MMP activity during AIA. A) Representative images showing MMP activity at day 21 after induction of AIA as determined by the presence of the neo-epitope VDIPEN with immunohistochemistry. B) *Apoe*⁻/⁻* mice showed a significant reduction of VDIPEN staining (44% lower), which was reduced by 63% in the patellofemoral area and by 60% in the tibiofemoral area. Horizontal and vertical lines represent the mean ±95% CI of 10 mice. Original magnification, 200x. * = \( P < 0.05 \)

**Apoe*⁻/⁻* mice show less cartilage destruction by reducing MMP activity in AIA**

We previously showed that activating FcγRs mediate MMP activation, which is crucial for degradation of cartilage in AIA.⁸,⁹ Because we found oxLDL to decrease the expression of FcγRI in macrophages, we next investigated whether high LDL-C levels during inflammation after induction of AIA in *Apoe*⁻/⁻* mice resulted in decreased MMP activity in the articular cartilage. We found a 52% reduction in the MMP-induced aggrecan neo-epitope VDIPEN in the cartilage of *Apoe*⁻/⁻* mice when compared to WT controls (mean arbitrary score 0,5±0,4 vs. 0,9±0,4, respectively) (Figure 5A), with a significant reduction in VDIPEN neo-epitopes both in the patellofemoral area (0,3±0,3 vs. 0,8±0,5) and the tibiofemoral area (0,6±0,6 vs. 1±0,6) (Figure 5B). In addition, we determined proteoglycan (PG) depletion and chondrocyte death as a further parameter for cartilage destruction. PG depletion and chondrocyte death were nearly absent in contralateral control knee joints (Figure 6A and 6C) and no differences were observed between *Apoe*⁻/⁻* mice and WT controls. In contrast, at day 21 of AIA, *Apoe*⁻/⁻* mice showed a significant reduction both in PG content and chondrocyte death in the articular cartilage (20% and 24% reduction, respectively) when compared to WT controls (Figure 6B and 6D). This indicates that high LDL-C levels by *Apoe* deficiency have a suppressive
effect on cartilage destruction only in combination with inflammation. However, although we observed a difference in PG content and chondrocyte death, both groups of WT and Apoe$^{-/-}$ mice did not show signs of cartilage erosions yet at this time point.

![Image](image_url)

**Figure 6.** Apoe$^{-/-}$ mice show reduced PG depletion and chondrocyte death during AIA. PG depletion and chondrocyte death were determined as a hallmark of cartilage degeneration in the patellofemoral and tibiofemoral areas. **A)** Contralateral control knee joints did not show signs of PG depletion neither in WT nor Apoe$^{-/-}$ mice. **B)** Arthritic Apoe$^{-/-}$ mice showed significantly less PG depletion as compared to WT controls. **C)** Contralateral control knee joints hardly showed any signs of chondrocyte death, which percentage was comparable between WT and Apoe$^{-/-}$ mice. **D)** Arthritic Apoe$^{-/-}$ mice showed significant reduction of chondrocyte death as compared to their arthritic WT controls. Horizontal and vertical lines represent the mean ±95% CI of 8 mice (contralateral control joints) or 10 mice (arthritic joints). Original magnification, 200 x. ** = $P < 0.01$.

**Discussion**

Several studies showed that dyslipidemia is present in RA patients $^{27,28}$. Active RA and chronic inflammation is accompanied by a reduction of total cholesterol (TC) and LDL-C serum levels $^{16,17}$. In contrast, early RA is marked by increased serum levels of TC and LDL-C $^{14,15}$. However, exactly how high levels of cholesterol influence onset and progression of RA remains poorly understood. In this study, we show that local induction of AIA in knee joints of hypercholesterolaemic Apoe$^{-/-}$ mice resulted in less joint inflammation and destruction of the articular cartilage during the course of arthritis.
Moreover, we show that the mechanistic basis can be a decreased expression of FcγRs on macrophages.

In our study we investigated Apoe"/- mice on a normal diet that develop elevated LDL-C levels that resemble those found in humans. We previously showed that the synovial lining macrophages are crucial in regulating both the onset and progression of AIA 4,29-31. Depletion of resident synovial macrophages prior to induction or during the course of arthritis completely prevented onset or continuation of arthritis 30,31. This is likely because the onset of AIA is initiated by intra-articular injection of mBSA that interacts with synovial lining macrophages 32,33 and the inflammation is driven by anti-mBSA antibodies forming ICs and their interaction with FcγRs on macrophages. In our study we found similar antibody titres of IgG subtypes against mBSA between Apoe"/- and WT mice. Apart from IgG mBSA-ICs, also IgG antibodies against oxLDL present in Apoe"/- mice, might contribute to disease activity 34. However, previous studies showed no association between these antibodies and development of atherosclerosis severity 35. This suggests that FcγR expression, rather than the level of ICs is altered by high cholesterol levels. In a naive joint FcγR expression on synovial lining macrophages is low 36. In contrast, in an inflamed synovium their expression is strongly enhanced 36 and the ratio between activating/inhibiting FcγRs expressed on macrophages contribute to accelerating inflammation.

Systemic inflammation is raised in Apoe"/- mice 37. APOE is largely produced by macrophages and its production is strongly up-regulated by TGFβ and down-regulated by cytokines such as IL-1β, TNFα, IFNy, and TLR4 ligands like LPS 38. APOE can modulate immune responses and act as an anti-inflammatory factor 39. However, we found that contralateral joints of Apoe"/- mice that were not injected with the antigen showed no signs of synovitis, indicating that in the absence of an IC trigger macrophages do not produce inflammatory mediators and that high LDL-C levels alone are not sufficient to induce joint inflammation. In addition, the absence of APOE in macrophages did not affect their levels of FcγRs, indicating that its absence does not skew the balance of these receptors and therefore does not alter their sensitivity for ICs. Further, arthritic mice showed a similar immune response and comparable production of pro-inflammatory cytokines in the arthritic sera. However, we cannot exclude that APOE exerts in vivo other immune-modulatory functions that may be responsible for the down-regulation of FcγRs during inflammatory arthritis.

Interestingly, oxLDL but not LDL significantly down-regulated activating FcγRs, particularly FcγRI that is crucial in driving oxidative burst by activation of the dihydronicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, leading to massive ROS production and MMP activation 40. To a smaller extent, oxLDL reduced the expression of activating FcγRIV, which aggravates inflammation in the AIA
model. Of note, the inhibiting FcγRIIb was also down-regulated upon both LDL and oxLDL. However, we assume that the stronger inhibition of activating FcγRI/IV overrules the modest reduction of inhibiting FcγRIIb, attenuating macrophage activation as a net result. LDL-C levels in the joint resemble those present in the blood. Upon entering an inflamed joint, LDL-C is oxidised by ROS into oxLDL and taken up by macrophages via scavenger receptors CD36, SR-A and LOX-1 and subsequently trafficked to lysosomes. OxLDL accumulation leads to massive cholesterol crystal formation, affecting lysosomal degradation of the FcγR-IC complex and recycling of FcγRs to cell membrane.

Previous studies using Apoe−/− mice showed conflicting data on the effect of high LDL-C levels on the development of collagen-induced arthritis (CIA). Asquith et al. showed that Apoe−/− mice were resistant to develop CIA, whereas Postigo et al. showed increased severity of CIA. In the serum transfer-induced arthritis model (STIA), Archer et al. found that Apoe−/− mice developed more synovitis and a stronger infiltration of foam macrophages after repeated injections of K/BxN serum when fed a western type diet, which favored the formation of atherosclerotic lesions. These lesions may increase the systemic production of inflammatory mediators and have a different effect on local joint inflammation, thus underlying the discrepancies with these findings. In our study, we did not observe foam macrophages in the synovium, probably due to the shorter duration of the model and administration of a normal diet. Interestingly, Archer et al. did not find differences in joint destruction. Although we did not corroborate the down-regulation of FcγR expression in the arthritic synovium, we find that oxLDL down-modulates macrophage FcγR expression, indicating it can be that its uptake by foam macrophages results in an even more pronounced decrease of FcγRs, thus reducing joint destruction.

Further, these controversial findings may be explained by the different genetic background of the Apoe−/− mice used. In a C57BL/6 (H2b) background, Apoe−/− mice were resistant against CIA, whereas Apoe−/− mice in a B10.RIII background showed increased pathology in the CIA model. Previously, we showed using an IC-arthritis model that mice in a B10.RIII background developed more inflammation than C57BL/6 mice due to different kinetics of FcγR expression. IC stimulation of macrophages derived from the B10.RIII strain, which is susceptible to developing autoimmune diseases, elicited a stronger up-regulation of activating FcγRI as compared to macrophages derived from C57BL/6 non-susceptible strain, whereas the expression of the inhibiting FcγRIIb was strongly down-regulated. In contrast, the up-regulation of activating FcγRI and down-regulation of inhibiting FcγRIIb were more prolonged in B10.RIII-macrophages after IC stimulation, which skewed the balance towards activating FcγRs. As we find that oxLDL uptake by C57BL/6-macrophages efficiently down-regulated the activating
FcγRs on their membrane, B10.RIII-macrophages may be less effective or even unable to regulate FcγRs after oxLDL uptake, which may explain the enhanced arthritis in \( \text{Apoe}^-/^- \) mice in a B10.RIII background. This study supports the hypothesis that previous controversial findings in \( \text{Apoe}^-/^- \) mice may be related to differences in the expression of FcγRs in the different strains.

Moreover, our findings underline the well known concept of the 'lipid paradox' in RA. Albeit dyslipidemia increases the incidence of atherosclerosis in RA patients, previous studies observed no correlation between high LDL levels and the development of RA. Furthermore, paradoxical outcomes have been described in RA patients concerning the effect of other metabolic and hormone-related factors. Adiponectin has been shown to act as a pro-inflammatory factor in the joints of RA patients, while they have an anti-atherogenic effect at the systemic level. Obesity, which is a major feature of the metabolic syndrome, is known to influence onset and progression of RA and has been shown to reduce the risk of developing RA in men, but not in women. Interestingly, a study by Turesson et al. described that high LDL-C predispose to RA in women, but not in men, pointing at a role for sex-specific hormones in modulating the effects of lipid on RA pathogenesis. In this study, we only used male mice. Therefore, it remains to be elucidated which effects high LDL-C levels may have on female mice. Because we observed reduced pathology in male \( \text{Apoe}^-/^- \) mice this may be partially due to the exposure to sex-related hormones.

In addition to synovitis, high LDL-C levels by \( \text{Apoe} \) deficiency, in combination with inflammation reduced cartilage destruction. IC binding to activating FcγRs on macrophages, particularly FcγRI, leads to abundant ROS production and plays a central role in activating latent MMPs in the articular cartilage, leading to breakdown of glycosaminoglycans and chondrocyte death. A previous study showed that lack of FcγRI associated with reduced MMP activation and cartilage destruction during AIA. OxLDL strongly reduced FcγRI levels in vitro, suggesting that high LDL-C levels and their oxidation in the inflamed joint may be responsible for the reduction of MMP activation and cartilage destruction observed in this IC-driven arthritis model in \( \text{Apoe}^-/^- \) mice. The presence of tissue inhibitor of MMPs (TIMPs) is important in reducing the levels of MMPs. However, as in our in vitro experiments the expression of TIMPs was not increased (data not shown), we assume it is unlikely that increased TIMP expression as the result of high LDL or oxLDL levels may be the cause of decreased MMP activity as determined by VDIPEN staining.

Apart from lowering activating factors like FcγRI, oxLDL uptake by macrophages leads to activation of anabolic factors like TGFβ. TGFβ can counteract the activity of pro-inflammatory cytokines like IL-1β, induce the expression of anti-inflammatory
cytokines like IL-10, up-regulate the inhibiting FcγRIIb and down-regulate the expression of the activating FcγRs.

Apart from affecting FcγR expression on macrophages, oxLDL may also target chondrocytes. However, we think it is unlikely that the reduced cartilage damage is mediated by a direct effect of oxLDL on FcγR regulation on chondrocytes, as the absence of either the activating or inhibiting FcγRs did not affect cartilage destruction in a model of experimental OA. Further, oxLDL can bind to LOX-1 on chondrocytes, increase the production of intracellular ROS and activate NF-κB, thus inducing a hypertrophic phenotype. Moreover, oxLDL binding to LOX-1 enhanced MMP3 production by chondrocytes, which may further contribute to cartilage pathology in RA. However, the data in our in vivo setting suggest that the suppressive effect induced by oxLDL on synovial macrophages may counterbalance oxLDL-mediated cartilage destruction as it was significantly decreased during the chronic phase of AIA. Collectively, our findings indicate that high levels of LDL/oxLDL by Apoe deficiency decreased inflammation and MMP-driven cartilage degeneration during onset and course of AIA, which is likely the result of oxLDL-driven FcγR down-regulation on synovial macrophages.

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**Disclosure statement** The authors disclose no competing interests.
References


Supplementary figure

Figure S1. Serum levels of pro-inflammatory cytokines are comparable in WT and Apoe⁻/⁻ arthritic mice. The levels of pro-inflammatory cytokines and chemokines were determined in the serum of WT and Apoe⁻/⁻ mice at day 21 of AIA. No significant differences were found in the production of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and KC as chemokine. Also IL-10, IFN-γ, MIP-1α and MCP-1 were measured, but all below the detection limit. Horizontal and vertical lines represent the mean ±95% CI of 6-10 mice.

Supplementary material

**Histological analysis**

Histological parameters (joint inflammation and cartilage damage) were scored in a blinded manner by two independent observers. Inflammation, defined as influx of inflammatory cells into synovium and joint cavity, was arbitrarily scored on a scale from 0 (no inflammation) to 3 (most observed inflammation). Cartilage destruction was assessed as PG depletion, scored arbitrarily from (no absence of PG) to 3 (complete absence of PG), and chondrocyte death, represented as the percentage of cartilage area containing empty lacunae with respect to the total area.

**Flow Cytometric analysis**

Alexa Fluor 647-labeled mouse anti-FcγR I (BD Pharmingen), Alexa Fluor 647–labeled rat anti-FcγR IIb (clone K9.361), PE-labeled rat anti-mouse FcγR III (R&D Systems) or APC-labeled Armenian hamster anti-FcγR IV (Biolegend). In addition, cells were labeled with Sytox Blue (Thermo Fisher Scientific) as viability dye to discriminate between live and dead cells. Samples were acquired using a Gallios flow cytometer, data were analyzed with Kaluza Analysis Software (Beckman Coulter Life Sciences) and values were presented as mean fluorescence intensity (MFI).
High LDL levels lessen bone destruction in antigen-induced arthritis by inhibiting osteoclast formation and function


[Epub ahead of print]
Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterized by severe joint inflammation and bone destruction as the result of increased numbers and activity of osteoclasts. RA is often associated with metabolic syndrome, whereby elevated levels of LDL are oxidized into oxLDL, which might affect osteoclastogenesis. In this study, we induced antigen-induced arthritis (AIA) in Apoe⁻/⁻ mice, which spontaneously develop high LDL levels, to investigate the effects of high LDL/oxLDL levels on osteoclast differentiation and bone destruction. Whereas basal levels of bone resorption were comparable between naive WT and Apoe⁻/⁻ mice, induction of AIA resulted in a significant reduction of bone destruction in Apoe⁻/⁻ mice as compared to WT controls. In line with that, the TRAP⁺ area on the cortical bone was significantly decreased. The absence of Apoe did affect neither the numbers of CD11b⁺Ly6C⁺high and CD11b⁻/Ly6C⁺high osteoclast precursors (OCPs) in the BM of naive mice nor their in vitro osteoclastogenic potential as indicated by comparable mRNA expression of osteoclast markers. Addition of oxLDL, but not LDL, to pre-osteoclasts from day 3 and mature osteoclasts from day 6 of osteoclastogenesis strongly reduced the number of TRAP⁺ osteoclasts and their resorptive capacity. This coincided with a decreased expression of various osteoclast markers. Interestingly, oxLDL significantly lowered the expression of osteoclast-associated receptor (Oscar) and the DNAX adaptor protein-12 encoding gene Tyrobp, which regulate the immunoreceptor tyrosine-based activation motif (ITAM) co-stimulation pathway that is strongly involved in osteoclastogenesis. Collectively, our findings suggest that under inflammatory conditions in the joint, high LDL levels lessen bone destruction during AIA, probably by formation of oxLDL that inhibits osteoclast formation and activity through modulation of the ITAM-signaling.
**Introduction**

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects the joints, and is characterized by synovial inflammation and excessive bone destruction \(^1,^2\). Elevated production of pro-inflammatory mediators leads to increased levels of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (RANK) ligand (RANKL), which induce the differentiation of myeloid precursors into osteoclasts \(^3,^4\) that is accompanied by increased expression of osteoclast-specific markers \(^5-^7\). In mice, particularly CD11b\(^+\)Ly6C\(^{high}\) and CD11b\(^-\)Ly6C\(^{high}\) precursors in the bone marrow (BM) are known to increase during experimental inflammatory arthritis and they are recruited to the inflamed joint where they can differentiate into osteoclasts \(^8,^9\). Next to RANK/RANKL signaling, the balance between the immunoreceptor tyrosine-based activation motif (ITAM) and the immunoreceptor tyrosine-based inhibitory motif (ITIM) co-stimulatory signaling plays an important role in modulating osteoclastogenesis \(^10^-^12\). Within this system, osteoclast-associated receptor (OSCAR) and paired immunoglobulin-like receptor A (PIR-A) activate ITAM-signaling via association with Fc receptor common γ-chain (FcRγ), whereas the triggering receptor expressed on myeloid cells-2 (TREM-2) activates ITAM-signaling upon binding to DNAX adaptor protein of 12 kDa (DAP12) \(^13,^14\). Conversely, the immunoglobulin-like receptor B (PIR-B) signals through an ITIM motif and as such negatively regulates the ITAM-signaling, and thus osteoclastogenesis \(^15\). As the activation of the ITAM-pathway may further drive osteoclast differentiation and function, its regulation can be particularly relevant in inflammatory immune-based diseases like RA \(^10\). RA is often associated with metabolic syndrome, and both are characterized by elevated levels of low density lipoproteins (LDL) that are oxidized into oxLDL in inflammatory environments \(^16^-^18\). Therefore, the enhanced presence of oxLDL during inflammatory arthritis might affect differentiation and activation of osteoclasts, causing bone destruction in the arthritic joints. In order to investigate this, we used Apolipoprotein E deficient (Apoe\(^{-/-}\)) mice that are commonly used as a model for hypercholesterolemia \(^19\). Apolipoprotein E (Apoe) is a lipoprotein that regulates LDL transportation and its absence strongly elevates circulating LDL levels in humans as well as in mice \(^20\). In this study, we elicited antigen-induced arthritis (AIA) in knee joints of Apoe\(^{-/-}\) mice and their wild type (WT) controls to investigate the effects of high LDL levels and their oxidation to oxLDL, increased by the inflammatory milieu, on osteoclast-mediated bone destruction. Further, using *in vitro* experiments, we aimed at identification of molecular pathways involved in the effects of LDL/oxLDL on osteoclast differentiation and activation.
Methods

Animals

*B6.129P2-Apoelm1Unc/J* mice (JAX strain) and wild type C57Bl/6J controls were obtained from the Charles River Laboratory (Leiden, the Netherlands). All mice were housed in filter-top cages and received a standard chow diet and acidified water *ad libitum*. Male mice between 10-12 weeks were used in all the experiments, which were performed in accordance with the Dutch regulations and guidelines for care and use of laboratory animals. All animal studies were approved by the Radboud University’s Animal Experiment Committee, Nijmegen-the Netherlands (RU-DEC 2014-191).

Induction of antigen–induced arthritis

Mice were immunized with 100 µg/mL of methylated bovine serum albumin (mBSA; Sigma–Aldrich) emulsified in 100 µl Freund’s complete adjuvant (CFA). Injections were divided over the flanks and the footpad of the front paws. Heat killed *Bordetella Pertussis* was administered as an additional adjuvant intraperitoneally. One week later, two subcutaneous injections of 50 µg of mBSA/CFA were given as a booster in the neck region. Two weeks after these booster injections, arthritis was induced in the right knee joint by intra-articular injection of 60 µg of mBSA in 6 µl saline.

Histology

Total knee joints were dissected, fixed in phosphate-buffered formalin (pH 7.4), decalcified in ethylenediaminetetraacetic acid (EDTA) and subsequently embedded in paraffin wax. Coronal sections of 7 µm of various depths, representing the whole knee joint, were stained with hematoxylin & eosin (H&E) for histological analysis. Bone destruction was evaluated in 13 well-defined areas of the knee joint (shown in Supplementary file 1) and scored arbitrarily on a scale from 0 (no erosion) to 3 (connection between joint cavity and bone marrow). Total knee joint sections were stained for tartrate-resistant acid phosphatase (TRAP), using the Leukocyte Acid Phosphatase Kit (Sigma-Aldrich) according to the manufacturer’s protocol. The amount of TRAP⁺ area present along the cortical bone and trabecular bone within the bone marrow (BM) cavity, representing osteoclasts, was measured in the arthritic joints as percentage (%) of positive area above a fixed threshold using Leica Application Suite software (Leica Microsystems).

Flow cytometry

Numbers of osteoclast precursors in the BM were determined with flow cytometry. Bone marrow was isolated from femurs and tibias of mice by flushing the marrow cavity
with medium and passing the cell suspension through a strainer. After lysis of
erthrocytes, bone marrow cells were incubated with Fc-blocking antibody (BD
Pharmingen antimouse CD16/CD32, clone 2.4G2; BD Biosciences, San Jose, CA, USA),
followed by staining with the following mix of antibodies: CD11b-fluorescein
isothiocyanate (FITC), CD90.2- phycoerythrin (PE), CD45R/B220-PE, CD49b-PE, NK1.1-
PE, Ly6G-PE, Ly6C-allophycocyanin-cyanine 7 (APC-Cy7), and F4/80-PE-Cy7 (all BD
Biosciences). Samples were acquired with a Gallios flow cytometer (Beckman Coulter
Life Sciences, Indianapolis, IN, USA).

In vitro osteoclast differentiation

Following BM isolation, total BM cells were seeded into 96-well plates at a density of
10^5 cells/well and cultured up to 7 days in 100 μl of α-minimum essential medium
(Thermo Fisher Scientific), supplemented with 5% FCS, penicillin/streptomycin, 30 ng/ml
recombinant mouse (rm)M-CSF, and 20 ng/ml rmRANKL (R&D Systems). Culture
medium was refreshed after 3 days.

oxLDL preparation and stimulation of osteoclasts

LDL was isolated by single-spin density gradient ultracentrifugation from EDTA-treated
blood from healthy volunteers and oxidized as previously described\(^\text{21}\). At various time-
points (day 3, 5 or day 6) of osteoclastogenesis, preosteoclasts or yet mature osteoclasts
were stimulated with either 10 μg/mL oxLDL or 10 μg/mL LDL-C as control and the
medium was refreshed every 3 days. This concentration was chosen to resemble the
LDL-C levels \(\text{in vivo}\)\(^\text{22}\).

RNA isolation and qRT-PCR

RNA from osteoclast cultures was isolated with TRIzol reagent (Sigma-Aldrich) at
different time-points during osteoclastogenesis (days 0, 3, 4, 6 and 7). Briefly, mRNA
concentration was measured with a Nanodrop spectrophotometer and subsequently
reverse-transcribed into complementary DNA (cDNA) using oligo (dT) primers. qRT-PCR
was performed using the Applied Biosystems StepOnePlus RT-PCR System (Thermo
Fisher Scientific). Specific primer sequences used to assess gene expression are listed
in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as
housekeeping gene. Samples were normalized for the expression of \(\text{Gapdh}\) by
calculating the comparative threshold:\(-\Delta Ct = -(C_{\text{gene of interest}} - C_{\text{Gapdh}})\).
Determination of TRAP activity in the supernatants

Culture supernatants were collected after 7 days of differentiation and TRAP activity was measured with a colorimetric assay. Briefly, p-nitrophenyl phosphate (NewEngland Biolabs) was diluted in buffer containing 420 mM acetic acid (Sigma-Aldrich) and 160 mM tartrate solution (Merck) and added 1:1 to culture supernatant. After 1 hour, the reaction was stopped with 0.5 M NaOH (Sigma-Aldrich) and the absorbance at 405 nm was determined using a spectrophotometric plate reader (Bio-Rad Laboratories).

Resorption assay on calcium phosphate–coated plates

Bone marrow cells were seeded at a density of $10^5$ cells/well onto 96-well biomimetic hydroxyapatite-like–coated plates that were prepared as previously described. Preosteoclasts or mature osteoclasts were stimulated with LDL/oxLDL starting from day 3 and day 5, respectively, and the medium was refreshed every 3 days. At day 10 the medium was removed from the wells, then 100 μl/well of distilled water was added to lyse the cells. To analyze pit-formation the coated layer was stained with a Von Kossa staining. Briefly, a 5% aqueous silver nitrate was added and incubated in the dark. Afterwards, wells were washed with water and incubated with 5% sodium carbonate in 4% formalin. Digital images were obtained with a camera mounted on an inverted-light microscope. The percentage of resorbed bone area was quantified using the Leica Application Suite software (Leica Microsystems).

Statistics

Statistics was performed using GraphPad Prism version 5.0 (GraphPad Software Inc.). Differences between two groups were tested using a two-tailed Student’s t-test for comparing parametric variables, Mann-Whitney test for non-parametric variables (e.g. histological score) and multiple comparisons were tested using One-way ANOVA followed by Bonferroni’s multiple comparison test. $P$ values <0.05 were considered significant. Data are presented as the mean ±SEM.
Results

_Apoe<sup>-/-</sup> mice show reduced bone destruction during AIA_

First, we determined bone destruction in knee joints of hypercholesterolemic _Apoe<sup>-/-</sup>_ mice and their WT controls during AIA. Histological analysis showed less severe bone erosion in arthritic _Apoe<sup>-/-</sup>_ mice as compared to their arthritic WT controls (Figure 1a). Quantification showed a significant reduction (by 25%) in bone erosion in _Apoe<sup>-/-</sup>_ mice (Figure 1b) when compared to WT mice. Only minor but comparable bone erosion was observed in naive joints of WT and _Apoe<sup>-/-</sup>_ mice (Figure 1c and 1d).

Figure 1. _Apoe<sup>-/-</sup>_ mice develop less bone destruction during AIA. a) Representative photomicrographs of bone erosion as determined by histology in arthritic _Apoe<sup>-/-</sup>_ mice and their wild type (WT) controls at day 21 after induction of antigen-induced arthritis (AIA). Original magnification, 200x. (F= Femur). b) Quantification of bone destruction showed that arthritic _Apoe<sup>-/-</sup>_ mice had a significant reduction of bone erosions as compared to WT controls. c) In contrast, naive knee joints of WT and _Apoe<sup>-/-</sup>_ mice showed minor but comparable erosions, due to physiologic bone remodeling. d) Quantification of bone remodeling in naive mice. Error bars represent the mean ±SEM of 10 mice (arthritic) and 8 mice (naïve). (** = P < 0.01 versus WT controls).

_Arthritic Apoe<sup>-/-</sup> mice develop less osteoclasts along the cortical bone_

Because we found less bone destruction in _Apoe<sup>-/-</sup>_ mice during AIA, we next determined by enzymehistochemical TRAP staining whether this was due to a lower number of multinucleated resorbing osteoclasts. In line with the decreased bone destruction, at day 21 of AIA we observed a significant reduction in the amount of TRAP<sup>+</sup> area (1.9-fold decrease) as well as the number of osteoclasts along the cortical bone of _Apoe<sup>-/-</sup>_ mice when compared to WT controls (Figure 2b and 2c). Further, we observed a different
morbidity of the osteoclasts present on the cortical bone, with the Apoe<sup>−/−</sup> cells being more flattened as compared to WT control cells (Figure 2a).

Figure 2. Induction of AIA in Apoe<sup>−/−</sup> mice results in the reduction of osteoclast numbers on the cortical bone. 

a) Representative photomicrographs of TRAP positive (TRAP<sup>+</sup>) cells along the bone surface in total knee joint sections of WT and Apoe<sup>−/−</sup> mice and WT controls at day 21 after induction of AIA. 
b) Quantification of TRAP<sup>+</sup> area along the cortical bone surface showed that arthritic Apoe<sup>−/−</sup> mice had a significant reduction of osteoclast number as compared to WT controls. 
c) Quantification of the number of TRAP<sup>+</sup> osteoclasts along the cortical bone. Error bars represent the mean ±SEM of 10 arthritic mice/group (** = P < 0.01 versus WT controls). Original magnification, 200 x.

**Apoe deficiency does not affect the number of osteoclast precursors in the bone marrow and their osteoclastogenic potential**

Next, we determined whether the absence of Apoe by itself could be the direct cause of the observed reduction in the number of osteoclasts. Flow cytometric analysis showed that the number of CD11b<sup>+</sup>Ly6<sup>C</sup><sub>high</sub> and CD11b<sup>+</sup>Ly6<sup>C</sup><sub>high</sub> cells, which are the main osteoclast precursors (OCPs) <sup>8, 9</sup>, were comparable in the BM of Apoe<sup>−/−</sup> and WT mice prior to induction of AIA. Interestingly, the percentage of CD11b<sup>−</sup>Ly6<sup>C</sup><sub>high</sub> cells in the BM was even significantly increased in arthritic Apoe<sup>−/−</sup> mice (Figure 3a and 3b). Next, we investigated whether the absence of Apoe itself altered their osteoclastogenic potential. We observed comparable kinetics in the expression of various osteoclast markers in differentiating osteoclasts from WT and Apoe<sup>−/−</sup> BM cells in vitro (Figure 3c). Underlining this finding, no significant differences were observed in the number of TRAP<sup>+</sup> and multinucleated osteoclasts obtained from WT and Apoe<sup>−/−</sup> cultures (58±10 vs. 52±7 osteoclasts, respectively) (Figure 3d). Moreover, we found no difference in the amount of TRAP<sup>+</sup> area between arthritic WT and Apoe<sup>−/−</sup> mice in the trabecular bone within the BM cavity, which is not directly in contact with inflammatory mediators in the arthritic synovium (Figure 3e and 3f). Together these data suggest that increased LDL oxidation in the inflammatory milieu rather than the Apoe deficiency in the arthritic joint may account for the differences in the number of osteoclasts on cortical bone.
Figure 3. The absence of Apoe does not affect the number of osteoclast precursors in the bone marrow or their osteoclastogenic potential. 

a) Comparable percentages of CD11b$^{+}$Ly6C$^{ hi}$ and CD11b Ly6C$^{ hi}$ osteoclast precursors (OCPs) were present in the bone marrow (BM) of naïve wild type (WT) and Apoe$^{-/-}$ mice (n=6 mice/group). 

b) Notably, whereas we observed decreased numbers of osteoclasts at day 21 after AIA induction, Apoe$^{-/-}$ mice showed a significantly higher percentage (%) of CD11b Ly6C$^{ hi}$ OCPs compared to WT mice.

c) Comparable messenger RNA (mRNA) expression levels of various osteoclast markers were determined at various time-points during osteoclastogenesis in WT and Apoe$^{-/-}$ bone marrow (BM) cells (n=6 mice/group). Colony stimulating factor 1 receptor (Csf1r); Tumor necrosis factor receptor superfamily member 11a (Tnfrsf11a); Nuclear factor of activated T cells 1 (Nfatc1); Dendritic cells specific transmembrane protein (Dcstamp); Acid phosphatase 5 (Acp5); Calcitonin receptor (Calcr); Chloride channel-voltage sensitive 7 (Clcn7); Carbonic anhydrase 2 (Ca2); Matrix metallopeptidase 9 (Mmp9); Cathepsin K (Ctsk).

d) Quantification of TRAP$^{+}$ osteoclasts shows no differences in the number of osteoclasts.

Moreover, as indicated in representative microphotographs, no differences in the amount of TRAP$^{+}$ osteoclasts were present in the trabecular bone within the BM. Original magnification, 200 x.

Quantification of the TRAP$^{+}$ area within the BM expressed as percentage. Error bars represent the mean ±SEM of 10 mice (* = P < 0.05 compared to WT controls).
oxLDL, but not LDL, blocks the differentiation of pre-osteoclasts into mature osteoclasts

Because the number of osteoclasts exclusively was reduced in the arthritic environment, we hypothesised that local inflammation may be involved. Apoe⁻/⁻ mice spontaneously develop elevated levels of LDL, and this LDL is oxidized into oxLDL in inflamed areas. Therefore, we investigated whether LDL or oxLDL affected in vitro osteoclast differentiation. First, at day 3 of differentiation pre-osteoclasts were stimulated with oxLDL or LDL for 24h and lipid uptake was detected by Oil Red O staining. As expected, oxLDL accumulated within the cells, whereas LDL did not. (Figure 4a). Pre-osteoclasts exposed to oxLDL for 24h showed a significant decrease of the mRNA levels of various osteoclast markers. In contrast, the levels of Adgre1, the gene encoding for F4/80 as macrophage marker remained significantly higher, underlining a blockage of osteoclast differentiation (Figure 4b). Next, to assess the effects of oxLDL on osteoclast formation, the cells were further cultured up to 7 days. In agreement with our findings on the mRNA level, oxLDL, but not LDL, determined a nearly complete inhibition (94% less than in control cells) of the number of TRAP⁺ and multinucleated cells (Figure 4c). This reduction was not due to cytotoxic effects of oxLDL, as indicated by the comparable fluorescence intensity signals after a DAPI staining among non-stimulated, LDL and oxLDL-stimulated cells (Figure 4d). Although also apoptotic cells can internalize the DAPI stain qualitative assessment of the DAPI positive cells showed no significant differences in the nuclear morphology (data not shown), suggesting that oxLDL does not affect viability.

oxLDL, but not LDL, inhibits the further fusion of multinucleated osteoclasts and their capacity to break down the bone mineral matrix

At day 6, mature osteoclasts were stimulated for 24h to investigate the effects of LDL and oxLDL at a later stage of differentiation. We found a significant reduction of TRAP⁺ and multinucleated cells upon oxLDL exposure (46% reduction as compared to control) (Figure 5a and 5b). In accordance with the inhibition of osteoclastogenesis, we found that also osteoclast–mediated degradation of biomimetic hydroxyapatite-like-coated plates was strongly and significantly impaired by oxLDL both when pre-osteoclasts and mature osteoclasts were stimulated (Figure 5c), indicating that exposure to oxLDL can inhibit the resorption machinery at different stages of osteoclastogenesis. Finally, as TRAP activity is commonly accepted as marker for bone resorption, we determined its secretion in the culture supernatants and found that exposure to oxLDL, but not LDL, of both pre-osteoclasts and mature osteoclasts significantly inhibited TRAP activity (Figure 5d).
Figure 4. oxLDL blocks the differentiation of mononuclear pre-osteoclasts into mature osteoclasts. Wild-type (WT) pre-osteoclasts were stimulated with 10µg/mL LDL or oxLDL for 24 h. a) Representative photomicrograph of pre-osteoclasts either non-stimulated (NS) and after exposure to LDL or oxLDL for 24h. Note that oxLDL, but not LDL, was internalized as determined by Oil Red O staining. Original magnification, 200x. b) The messenger RNA (mRNA) levels of various osteoclast-related genes were significantly down-regulated in WT pre-osteoclasts after exposure to oxLDL, whereas Adgre1 was significantly increased. c) Photomicrographs and quantification of TRAP positive (TRAP⁺) cells show a strong inhibition of osteoclastogenesis upon oxLDL. Original magnification, 100x. d) Of note, oxLDL did not affect cell viability as determined by fluorescence intensity (FI) signals of DAPI stain. Error bars represent the mean ±SEM of 6 mice. (* = P < 0.05, ** = P < 0.01, *** = P < 0.001 versus NS control; # = P < 0.05, ## = P < 0.01 compared to LDL stimulated samples).
Figure 5. oxLDL strongly restrains the formation of multinucleated osteoclasts and their resorptive activity at a later stage of differentiation. Wild-type (WT) multinucleated osteoclasts were stimulated with 10µg/mL LDL or oxLDL for 24h. a) Representative photomicrographs and quantification of TRAP positive (TRAP*) cells show a strong inhibition of osteoclastogenesis upon oxLDL, but not LDL stimulation. Original magnification, 100x b) Note that oxLDL did not affect cell viability as determined by fluorescence intensity (FI) signals of DAPI stain (n=6 mice). c) Representative photomicrographs and quantification of resorption on biomimetic hydroxyapatite-like-coated plates show a significant reduction of pit areas upon exposure of both pre-osteoclasts (day 3) and multinucleated osteoclasts (day 5) to oxLDL; of note, exposure to LDL reduced pit formation, though this reduction was not significant (n=6 mice). Original magnification, 100x d) Finally, TRAP activity in the culture supernatants of WT mature osteoclasts was strongly down-regulated.
both upon exposure of pre-osteoclasts (day 3) and multinucleated osteoclasts (day 5) to oxLDL, but not LDL (n=3 mice). Error bars represent the mean ±SEM. (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to NS controls; ## = $P < 0.01$, ### = $P < 0.001$ compared to LDL stimulated samples).

**oxLDL strongly down-regulates the expression of molecules involved in the ITAM co-stimulation pathway**

To identify a possible mechanism responsible for the effects of oxLDL on osteoclasts, we determined whether oxLDL affected the RANK/RANKL signaling, which is crucial to induce osteoclastogenesis. We found that oxLDL stimulation of pre-osteoclasts did not change the mRNA levels of *Tnfrsf11a* and *Csf1r* encoding genes for RANK and M-CSFR, respectively (Figure 6a). Next to RANK/RANKL signaling, factors involved in the co-stimulatory pathway play an important role in stimulating osteoclastogenesis and bone destruction. Therefore, we addressed whether oxLDL affected the expression of these factors. Interestingly, the mRNA of the immunoreceptor Oscar was strongly and significantly down-regulated in pre-osteoclasts by oxLDL. Although the expression of *Trem2* was significantly increased, we found that the levels of *Tyrobp*, a gene that codes for DAP-12, which binds to TREM2, were significantly down-regulated upon oxLDL exposure. In contrast, the expression of other factors of the ITAM/ITIM co-stimulatory pathway such as *Pira*, *Fcrg* and *Pirb* - did not change upon oxLDL (Figure 6b). Further, we assessed the expression of Fcγ receptors (FcγRs) as they are involved in ITAM-mediated induction of osteoclastogenesis and found that oxLDL significantly up-regulated the mRNA of *Fcgr3*, which has been associated with the inhibition of osteoclastogenesis (Figure 6c). These data suggest that at later stages of osteoclastogenesis, oxLDL does not affect either the RANK/RANKL signaling or the response to M-CSF-induced differentiation of pre-osteoclasts, but rather targets the ITAM co-stimulatory pathway to inhibit the further differentiation of pre-osteoclasts into mature and functional cells.
Figure 6. oxLDL down-regulates the mRNA levels of molecules of the ITAM-pathway in pre-osteoclasts. 

a) Wild-type (WT) pre-osteoclasts stimulated with LDL or oxLDL for 24 h showed comparable mRNA expression levels of RANK and M-CSF encoding genes Tnfrsf11a and Csfr1. b) Interestingly, oxLDL affected the mRNA levels of various molecules involved in the ITAM-signaling pathway, particularly it strongly down-regulated Oscar and DAP-12 encoding gene Tyrobp. c) Finally, of note, exposure to oxLDL significantly up-regulated the mRNA expression of Fcgr3. Error bars represent the mean ±SEM of 4 mice. (** = P < 0.01, *** = P < 0.001 compared to NS controls; # = P < 0.05, ## = P < 0.01, ### = P < 0.001 compared to LDL stimulated samples).
### Table 1

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Discussion

In the present study, we show that high LDL levels in Apoe−/− mice in combination with local inflammation resulted in decreased bone destruction and reduction of the number of osteoclasts in the knee joints after local induction of AIA. Further, we show that oxLDL but not LDL prevents the differentiation of osteoclasts, and their function probably by decreasing the activity of the ITAM co-stimulatory pathway.

RA is often accompanied by dyslipidemia, characterized by high levels of LDL and low levels of high density lipoprotein (HDL) cholesterol and LDL has been proposed as a novel serum marker to predict radiographic progression in RA patients. However, the relation between high LDL levels and bone erosion in RA is still a matter of debate. In the serum transfer-induced arthritis (STIA) model Archer et al. found that Apoe−/− mice fed a western-type diet (WD) developed enhanced synovial inflammation, although bone destruction was not affected. In line with this, a previous study from our lab showed increased early ectopic bone formation in dyslipidemic mice during experimental osteoarthritis, likely due to the enhanced production of transforming growth factor-beta (TGF-β) by synovial macrophages. TGF-β strongly inhibits osteoclast formation and activity. As we found that elevated levels of LDL and their increased oxidation during AIA in the Apoe−/− mice decreased bone destruction it can be that oxLDL acts as an anabolic factor by inducing the local production of TGF-β, thus decreasing osteoclast formation and bone destruction. Therefore, further studies including intra-articular injections of oxLDL will help to shed light on the effect of oxLDL on the development of joint destruction.

In RA patients the permeability of the endothelium is altered which allows a substantial access of LDL from the blood stream to the subendothelial layer and joint tissues, and as such LDL levels in synovial fluids of RA patients resemble those in the blood. After entering the inflamed joint, LDL becomes oxidized to oxLDL through the action of pro-inflammatory cytokines that stimulate the release of reactive oxygen species by inflammatory cells (ROS). Our findings show that hypercholesterolemic Apoe−/− mice developed less bone destruction owed to a substantial reduction of the number of osteoclasts in the arthritic knee joint. Apoe−/− mice are a widely accepted mouse model for hypercholesterolemia. Apart from regulating LDL levels, APOE has been described as an inhibitor of various immune cells and its absence may stimulate systemic inflammation thereby potentiating bone erosion. However, studying Apoe−/− mice with high LDL levels, only physiological bone remodeling was found in naive knee joints not different from that in WT mice. In addition, in the absence of an inflammatory trigger, APOE did not affect the percentage of OCPs present in the bone marrow nor the kinetics of expression of osteoclast markers and their osteoclastogenic potential. This in
accordance with a previous study by Schilling et al. that described that mice lacking Apoe displayed an increased trabecular bone volume caused by an increased bone formation rate, probably due to undercarboxylation of osteocalcin that is an inhibitor of osteoblastogenesis. Similarly, Nikolov et al. reported that Apoe<sup>−/−</sup> mice had higher cortical and trabecular bone mass than control WT mice at the basal level due to increased osteoblast-mediated bone formation. However, it has to be noted that in contrast to our study in which we used young, 12-weeks old, male mice they used 20-weeks old female mice, suggesting that under hypercholesterolemic conditions both sex and age have an additional effect on bone metabolism. In our study we showed that neither high levels of LDL alone nor Apoe deficiency by itself, promote osteoclastogenesis and bone erosion. Further, increased LDL oxidation in arthritic Apoe<sup>−/−</sup> mice did not lead to a reduction of bone mass as determined by measuring the surface of the cortical bone in the arthritic knee joints (data not shown). Together, this suggests that Apoe deficiency and high LDL levels can interfere with osteoblastogenesis and bone formation, whereas bone resorption remains unaffected. In contrast, LDL receptor deficient (Ldlr<sup>−/−</sup>) mice, which represent another model of hypercholesterolemia, showed increased bone mass caused by impaired osteoclast-mediated bone resorption, which may be due to the fact that the LDL receptor is required to allow cholesterol uptake into cells under physiologic conditions.

Some studies have described that hypercholesterolemia and increased lipid oxidation reduce bone mass. Discrepancies between our and previous findings may be explained by the fact that we studied Apoe<sup>−/−</sup> mice that received a standard chow diet, while these previous studies used a high fat diet (HFD). HFD massively elevates LDL levels and its oxidation, thus contributing to increase of systemic inflammation that can affect bone formation.

Interestingly, although LDL levels as found in Apoe<sup>−/−</sup> mice fed a standard chow diet do not change bone destruction in a normal joint, in combination with synovial inflammation a strong reduction in development of bone destruction was observed that coincided with a significant reduction of osteoclasts on the cortical bone. This reduction was not due to a difference in OCP numbers since the numbers of CD11b<sup>+</sup>Ly6C<sup>high</sup> and CD11b<sup>+</sup>Ly6C<sup>high</sup>, the main OCPs, were not decreased in BM of arthritic Apoe<sup>−/−</sup> mice and their WT controls. This suggests that even though Apoe<sup>−/−</sup> mice developed decreased synovial inflammation the generation of OCPs in the BM and their potential differentiation into osteoclasts in the knee joints is not affected. Interestingly, bone areas within the same knee joint that did not have direct contact with inflammatory cells (like the BM cortical bone) showed a similar amount of TRAP<sup>+</sup> osteoclasts between the two strains again indicating that high LDL levels alone without oxidation do not impair the number of osteoclasts. Therefore, we assume that enhanced LDL oxidation in the
arthritic synovium of Apoe−/− mice may lead to a locally induced reduction of osteoclast numbers and diminished bone destruction.

As we could not detect oxLDL directly within the inflamed knee joints as the heterogeneity of oxLDL, due to different degrees as well as specific epitopes of oxidation, makes its detection in vivo very difficult, we further investigated the effect of LDL/oxLDL in an in vitro osteoclastogenesis assay. Our findings strongly point out that oxLDL prevented the differentiation into multinucleated osteoclasts both when given in an early and later stage of osteoclastogenesis, thereby strongly reducing gene expression of osteoclast markers and resorption on biomimetic hydroxyapatite-like–coated plates. The decrease in osteoclastogenesis was not due to cell death since DAPI staining showed comparable numbers of cells in treated versus non-treated groups. In contrast to oxLDL, we observed no significant in vitro effect of LDL on osteoclast formation and activity and LDL only mildly reduced resorption. The latter may also be an oxLDL effect since the extended time frame of the cultures needed to investigate the resorption may have led to a partial oxidation of native LDL into oxLDL in the culture supernatant. Our findings are in line with the study of Maziere et al. who also found that oxLDL blocks the in vitro differentiation of OCPs into osteoclasts. However, in contrast to their studies in which oxLDL was added to very early stages of OCPs we focused more on further differentiated pre-osteoclasts. The reason for this is that OCPs coming from the BM that enter the arthritic joint probably exhibit a more developed differentiation stage that is further steered by the inflammatory process within the inflamed synovium. Interestingly, Maziere et al. found that oxLDL blocks the in vitro differentiation of BM OCPs into osteoclasts upstream by inhibiting the RANK/RANKL signaling axis, which is crucial for induction of osteoclastogenesis. In contrast, we found that stimulation with oxLDL when added later on in the differentiation process of osteoclastogenesis had no effects on the expression of RANK, but rather on some of the factors involved in the likewise important ITAM-mediated signalling pathway. The co-stimulatory pathway for osteoclastogenesis is triggered via phosphorylation of the ITAM domain present on the adapter molecules γ-chain which is paired with the receptors OSCAR, PIRA and FcyRs and DAP12 with TREM2. Of note, Oscar mRNA levels were sharply down-regulated in pre-osteoclasts exposed to oxLDL, implying that fewer OSCAR receptors are available to trigger the ITAM-pathway through the γ-chain. In contrast, Trem2 mRNA was significantly up-regulated by oxLDL. However, as we find that its signaling molecule Tyrobp is strongly decreased, it is unlikely that the up-regulation of Trem2 leads to a greater activation of the co-stimulatory pathway of osteoclastogenesis.

Previous studies reported that TREM-2 expression steers the differentiation of M2-like macrophages. Moreover, pre-osteoclasts exposed to oxLDL also showed increased mRNA levels of F4/80 which is a major macrophage marker. These results indicate
that cells exposed to oxLDL differentiate toward the macrophage rather than the osteoclast lineage despite high levels of RANKL and M-CSF. Moreover, FcγRIII, which has been shown to inhibit osteoclastogenesis by sequestering the γ-chain and withholding its association with osteoclast-inducer immune receptors like OSCAR, was significantly up-regulated. It has to be mentioned that in this study we predominantly focused on the ITAM co-stimulatory signaling pathway for osteoclastogenesis and therefore cannot rule out that oxLDL interferes with other signaling pathways (i.e.: IL-6, TNF-α) that are relevant for bone destruction in RA. Together, these observations substantiate the hypothesis that at later stages of osteoclastogenesis, oxLDL inhibits the differentiation into mature cells by interfering with the ITAM co-stimulatory pathway.

In another study, Dawodu et al. showed that oxLDL stimulation of human OCPs from peripheral blood impaired osteoclast activity via inhibition of CtsK secretion. In our study, we also found that Ctsk but also Mmp9 mRNA levels were sharply decreased and may contribute to lowering of bone erosion. This makes it highly likely that oxLDL protects from bone erosion by inhibition of resorption of both inorganic and organic matrix.

Conflicting results have been reported in RA patients following cholesterol-lowering therapies. Whereas the use of statins is beneficial in managing the cardiovascular (CV) risk in RA patients, several studies have described the risk of developing RA during statin treatment in subjects prone to develop RA. In line with this, experimental studies found that mice treated with statins showed accelerated onset of disease in a collagen-type II-induced arthritis (CIA) model. Our study supports the hypothesis that lowering LDL levels may boost both initiation and progression of bone destruction locally within an inflamed joint.

In summary, our findings suggest that high levels of LDL in combination with inflammation in the knee joint leads to oxLDL that inhibits bone destruction probably by suppressing osteoclast differentiation via decreased activation of the ITAM co-stimulatory pathway. Our study suggests that LDL-lowering therapies that are often used to reduce CV risk in RA patients should be used with caution since lowering of local levels of LDL within the inflamed joints may diminish the suppressing mechanism induced by oxLDL leading to increased osteoclastogenesis and bone destruction.

**Acknowledgements**

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Disclosure statement The authors disclose no competing interests.

References


Supplementary File 1. Graphical representation of bone erosion scoring system

P= Patella; F= Femur; L= Cruciate ligament; T= Tibia
Human APOE4 results in more severe experimental osteoarthritis in comparison to APOE3; APOE-isoforms as risk factor for osteoarthritis development

Manuscript in preparation
Abstract

**Background**: Apolipoprotein E (APOE) is particularly known for its role in promoting cholesterol metabolism. Recent studies, however, have shown that this molecule is also capable of suppressing innate immunity. In humans different polymorphisms of APOE exist, all with different anti-inflammatory efficacies. A role for APOE-isoforms in innate immune diseases such as Alzheimer’s disease and multiple sclerosis has already been described. In this study we investigate whether APOE polymorphisms could also play a role during osteoarthritis (OA) by comparing disease severity in mice transgenic for human APOε3 and APOε4 in a experimental OA model.

**Methods**: OA was induced by intraarticular injection of collagenase in B6.129P2-Apoε<sup>tm2(APOE*3)Mae</sup>N8 (APOε3) and B6.129P2-Apoε<sup>tm3(APOE*4)Mae</sup>N8 (APOε4) mice. Forty-two days after OA-induction knee joints were isolated and used for histological analysis.

**Results**: Naïve knee joints of APOε3 and APOε4 mice showed no abnormalities, nor were there any histological differences between the two groups. After induction of OA, however, APOε4 mice developed 2.3 times more synovial thickening than APOε3 mice ($P = 0.004$). Furthermore 32% more cartilage damage ($P = 0.019$) and an average of 2.6 times more ectopic bone formation was observed in APOε4 mice compared to APOε3 mice.

**Conclusions**: This brief study shows that mice with the human APOε4 gene develop a more severe OA compared to mice with the human APOε3 gene. Although the focus of this study is exclusively on histological data of an experimental OA model, it hints towards a possible role of APOE polymorphisms in human OA development and offers a possible new risk factor for this disease.
CHAPTER 6

Introduction

Osteoarthritis (OA) is a debilitating disease with a high worldwide prevalence. Although modulation of chondrocytes, leading to extracellular matrix and eventually cartilage damage, is seen as the most important factor for initiating the disease \(^1\), interplay with the synovium is undeniably important for aggravation of OA pathology \(^2\). Previously, we have shown that synovial macrophages are important in different OA processes such as inflammation \(^3\), cartilage destruction \(^4\) and osteophyte formation \(^5\) during both human and experimental OA. Activated synovial macrophages are major producers of damage-associated molecular patterns S100A8 (myeloid-related protein-8) and S100A9 (myeloid-related protein-14). These so-called alarmins are released in large amounts upon synovial activation, aggravating joint inflammation \(^6\) and playing a pivotal role in the development of osteoarthritis \(^7\).

Apart from pro-inflammatory factors, synovial macrophages also release anti-inflammatory factors, thereby controlling synovial activation. Apolipoprotein E (APOE) is such a protein which is produced in high amounts by the liver, but also by macrophages. Macrophage-activation strongly upregulates APOE production. Although this class of apolipoprotein was initially recognized for transporting lipoproteins and thereby promoting cholesterol metabolism, it has, more recently, also shown to play an important role in immunity \(^8\). APOE can modulate innate immunity by suppressing production of proinflammatory cytokines \(^9\), as well as by inhibiting oxidation of low-density lipoproteins (LDL), thereby preventing formation of pathogenic oxidized LDL \(^10\). Furthermore, APOE-signaling via the very low density lipoprotein receptor and APOE-receptor 2 has shown to promote macrophage conversion from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype \(^8\). In humans, three polymorphisms of APOE exist, namely APOε2, APOε3 and APOε4, which have different efficacies in suppressing innate immunity (ε2>ε3>ε4). In light of this immunomodulating function of APOE, specifically the APOε4-genotype has been associated with inflammatory diseases such as atherosclerosis, Alzheimer’s disease and multiple sclerosis \(^11,12\). Since innate immunity also plays an important role in OA-pathology, we investigate in this concise report the role of APOε4 in experimental OA using mice with targeted homozygous replacement of the endogenous mouse APOE-gene by the human APOE3 or APOE4 gene.
Methods

Animals

Twelve weeks old female B6.129P2-Apoetm2[APOE*3]MaeN8 (APOε3) and B6.129P2-Apoetm3[APOE*4]MaeN8 (APOε4) targeted replacement mice were derived from Taconic (Hudson, NY, USA) and were held in filter-top cages and received food and water ad libitum. OA was induced by intra-articular injection of 1 unit bacterial collagenase (Sigma-Aldrich, St. Louis, MO, USA) into the right knee joint on day 0 and day 2. Mice were sacrificed on day 42, after which knee joints were collected for histology. Serum was obtained for colorimetric determination of cholesterol. This study was approved by the Institutional Review Board (Animal Experiment Committee Radboud University) and was performed according to the related codes of practice.

Histology

Tissue was fixed in 4% buffered formalin, decalcified in formic acid and embedded in paraffin. Eight representative sections (7 μm) of various depths per joint were mounted on glass slides and stained with H & E or Safranin O-fast green for histological analysis. Sections were randomly coded and scored in a blinded way. Synovial thickening was measured using an arbitrary scoring system (0 = no synovial thickening; 1 = lining of two cell layers; 2 = several extra cell layers; 3 = clear inflammation with cell infiltrate or exudate). Cartilage damage in the tibial-femoral joint was scored using the Pritzker OA score, adapted for mice (from 0 = no damage to 30 = maximal damage)\(^{13}\), and size of ectopic bone formation by digital image analysis (Leica Application Suite v4.1; Leica Microsystems, Wetzlar, Germany). The mean of three (synovial thickening, ectopic bone formation) or five (cartilage damage) sections of different depths was determined per knee joint.

Statistics

Differences in pathology between APOε3 and APOε4 mice were statistically tested using a Mann Whitney U test (synovial thickening, cartilage damage) or a student’s t-test (size ectopic bone formation). All analyses were performed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data are depicted as mean ± 95% confidence interval.
Results

To investigate whether different human APOE-isoforms can affect OA pathology, we used *APOε3* and *APOε4* transgenic mice in this study. Under normal conditions and a conventional diet, these mice have no abnormal plasma cholesterol levels\(^{14}\). Also in this study we found no differences in cholesterol levels between the two groups (Figure 1).

![Figure 1](image)

**Figure 1.** *APOε3* and *APOε4* mice show comparable levels of total cholesterol and LDL cholesterol. Serum of both groups was analyzed for total cholesterol (A) and LDL-cholesterol (B) concentrations. \(n = 10\) mice per group; data are presented as mean ± 95% confidence interval.

In naïve knee joints of both *APOε3* and *APOε4* transgenic mice we found no obvious pathology at the age of 12 weeks and there was no difference in synovial thickness between the two species (Figure 2A). We induced OA by intraarticular injection of collagenase and 42 days after OA-induction, the synovial lining layer in *APOε4* mice was significantly thickened compared to *APOε3* mice (from 0.8 to 1.8; Figure 2B and D). Over time, OA pathology developed as expected in both strains, resulting in an moderate-severe score for cartilage damage between 10 and 20 (on a scale of 30). At the lateral site of the tibia, *APOε4* mice had significantly more cartilage damage compared with *APOε3* mice (16 and 11, respectively). An increase in cartilage damage was also seen in other cartilage plains, resulting in a significantly higher total score for cartilage damage in *APOε4* mice compared with *APOε3* mice (13 and 11, respectively; Figure 2C and D).
Also on a third hallmark of OA, ectopic bone formation, we found differences between APOε3 and APOε4 mice. We scored size of ectopic bone on six different locations using digital image analysis (Figure 3B). All six locations showed increased ectopic bone size in APOε4 mice in comparison with APOε3 mice with an average fold increase of 2.6. In particular, the lateral (39397 μm² versus 7736 μm²) and medial (16974 μm² versus 5739 μm²) side of the femur and the medial side of the tibia (264702 μm² versus 111676 μm²) showed significantly more ectopic bone formation in APOε4 mice (Figure 3A and C).

Figure 2. APOε4 mice show increased synovial thickening and cartilage destruction in comparison to APOε3 mice during experimental OA. Naive knee joints of both groups show no significant synovial thickening (A). Forty-two days after induction of OA, APOε4 mice had more synovial thickening (B) and cartilage damage (C) compared to APOε3 mice. Panel D shows representative photomicrographs of synovial thickening and cartilage damage. n = 4 mice per group for naïve joints and n = 10 mice per group for OA knee joints; data are presented as mean ± 95% confidence interval.
Discussion

This brief report is the first to describe a relation between APOE polymorphisms and OA pathology. Differences between APOε3 and APOε4 in the ability to suppress innate immunity have been reported and are associated with several diseases in which innate immunity plays a role\textsuperscript{11,12}.

In this study, we used an experimental model for osteoarthritis, characterized by synovial activation-mediated joint pathology, to show that different isoforms of APOE affect the disease outcome. Our data show that mice in which the murine APOE is replaced by human APOε4 develop a more severe OA compared with mice that have
murine APOE replaced by APOε3. Since innate immunity plays an important role in OA pathology \(^3\) and APOE acts as an immunosuppressive protein \(^8-10\), the lack of immunosuppressive capacity of human APOε4 could be an explanation for the increased pathology we observed. Even though, at this time, we merely focus on histological data from an experimental model, it gives us early evidence to support the hypothesis that specific APOE polymorphisms in OA patients affect disease outcome.

Since, in this study, we only compare the two groups (APOε3 and APOε4) with each other, we cannot draw any conclusions on whether APOε4 is aggravating pathology, or APOε3 is protective against OA pathology. Unfortunately, this will be difficult to test in the present \textit{in vivo} system, as proper control groups for this specific question will be hard to find. Using WT mice would only show differences between human and murine APOE and using APOE-deficient mice would lead to all sorts of additional pathology due to increased systemic cholesterol levels \(^{15}\).

In our opinion, discussing these preliminary data is an important first step in identifying the APOE molecule and its different isoforms as potential player in OA pathology. Even though the exact impact of different APOE-isofoms on OA is yet to discover, this brief report hints towards a possible role for APOε4 in human OA development and posits the APOE-genotype as a new risk factor for this disease.

Although we only focus in this study on disease severity and not on disease initiation, it would be of interest to investigate whether the APOε4-genotype would give a higher chance to develop inflammatory OA compared with the APOε3 or APOε2-genotype, using human cohort studies.
References

Genetic modification of ER-Hoxb8 osteoclast precursors using CRISPR/Cas9 as a novel way to allow studies on osteoclast biology


* These authors equally contributed

Abstract

Osteoclasts are cells specialized in bone resorption. Currently, studies on murine osteoclasts are primarily performed on bone marrow–derived cells with the use of many animals and limited cells available. ER-Hoxb8 cells are conditionally immortalized monocyte/macrophage murine progenitor cells, recently described to be able to differentiate toward functional osteoclasts. Here, we produced an ER-Hoxb8 clonal cell line from C57BL/6 bone marrow cells that strongly resembles phenotype and function of the conventional bone marrow–derived osteoclasts. We then used CRISPR/Cas9 technology to specifically inactivate genes by biallelic mutation. The CRISPR/Cas9 system is an adaptive immune system in Bacteria and Archaea and uses small RNAs and Cas nucleases to degrade foreign nucleic acids. Through specific-guide RNAs, the nuclease Cas9 can be redirected toward any genomic location to genetically modify eukaryotic cells. We genetically modified ER-Hoxb8 cells with success, generating NFATc1−/− and DC-STAMP−/− ER-Hoxb8 cells that lack the ability to differentiate into osteoclasts or to fuse into multinucleated osteoclasts, respectively. In conclusion, this method represents a markedly easy highly specific and efficient system for generating potentially unlimited numbers of genetically modified osteoclast precursors.
CHAPTER 7

Introduction

Osteoclasts are specialized cells that are unique in their ability to dissolve organic and inorganic components of bone. This tissue undergoes continuous remodeling throughout life and shows a tightly coordinated balance between degradation and synthesis. Alteration of this process could result in perturbations in skeletal structure and function and potentially in morbidity, such as osteoporosis, inflammatory arthritis, and periodontitis. Because of their key role in the physiology and pathology of bone metabolism, there is strong interest in understanding the complex biology of these cells. Nowadays murine experimental osteoclasts are commonly derived from spleen or BM cells under the influence of M-CSF and RANKL, with a time-consuming isolation procedure, few cells obtainable, and a scarce possibility of genome editing. The use of a stable cell line that strongly resembles primary cells would aid in overcoming these limitations and simplify genome-editing approaches. Until now, different methods have been adopted to develop immortalized cell lines that are able to differentiate into functional osteoclasts. Either transgenic mice with an SV40 large tumor antigen or p53 knockout have been used to directly isolate immortalized osteoclast precursors cells from the animal. In vitro immortalized progenitor cell lines and preexisting or new macrophage-like cell lines have also been used to generate osteoclasts (MOCP-5, TMC16, ML-6 and ML-7, and BDM-1). However, these cell lines have limitations or are not widely used. Some of the described cell lines (bcl-XL/Tag cells, macrophage-like cell lines C7, and BDM-1) require a coculture system with stromal cells or primary osteoblasts, increasing the complexity of control and study of the differentiation process. Other cell lines, such as ML-6 and ML-7, differentiate into TRAP+ cells but fail to form multinucleated giant cells. Finally, the RAW 264.7 cell line is broadly used to obtain osteoclasts in vitro; however, the endogenous production of M-CSF by these cells differs largely from the primary osteoclast culture. The ER-Hoxb8 cell line is a murine, conditionally immortalized, monocyte precursor cell line that has recently been shown to differentiate toward functional osteoclasts in vitro. Hoxb8 is a member of the Homeobox gene family, a family of transcription factors that controls various aspects of development. Some members, including Hoxb8, have been shown to counteract myeloid differentiation, providing an explanation for their role in human and mouse myeloid leukemia. In ER-Hoxb8 cells, Hoxb8 is fused to the ERHBD as an engineered, chimeric protein (Hoxb8-ERHBD). Stably transduced progenitor cells expressing Hoxb8-ERHBD can be expanded in the presence of β-estradiol and GM-CSF to obtain essentially unlimited numbers of monocyte/macrophage-committed precursors, which can be differentiated toward specific types of cells upon removal of estrogen and consequent silencing of the chimeric Hoxb8 protein. This cell line represents a useful tool for obtaining unlimited numbers of osteoclasts and offers the possibility of genetic modification. In the present study, we describe a method to produce genetically...
modified, ER-Hoxb8–derived osteoclasts using CRISPRCas system technology. The CRISPR/Cas system is an adaptive immune system in bacteria and Archaea that uses small RNAs and Cas nucleases to degrade foreign nucleic acids of invading viruses and plasmids. Among the 3 different CRISPR/Cas systems discovered, the type II CRISPR/Cas9 system has been the best characterized and optimized to induce site-specific DNA double-stranded breaks in eukaryotic cells. By providing a 20-nucleotide single gRNA complementary to a DNA fragment near a PAM sequence, the nuclease Cas9 can be directed toward any genomic region of interest. In the present study, we developed a clonal ER-Hoxb8 cell line from WT C57BL/6 mice, showing comparable characteristics to BM-derived primary osteoclasts that can be used for generating specific gene deletions using the lentivirus CRISPR-Cas9 system.

Materials and Methods

Isolation, cloning, and culture of ER-Hoxb8 conditionally immortalized precursors

BM cells were isolated from femurs and tibias of C57BL/6 WT mice. ER-Hoxb8 conditionally immortalized precursors were generated, as previously described. The ER-Hoxb8 cells obtained were subcloned by limiting dilution in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FCS (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), gentamicin (Centrafarm Nederland, Etten Leur, the Netherlands), 1 μM β-estradiol (Sigma-Aldrich, St. Louis, MO, USA), and 1% conditioned medium from GM-CSF-producing B16 melanoma cells expressing murine colony-stimulating factor 2 cDNA. Single clones were expanded in the same medium.

Differentiation of ER-Hoxb8 conditionally immortalized precursors toward osteoclasts and macrophages

ER-Hoxb8 cells were washed in RPMI 1640 medium to completely remove β-estradiol. For differentiation, ER-Hoxb8 was seeded in 96-well tissue culture–treated plates at a density of 50,000 cells/well in 150 μl of α-MEM medium (Thermo Fisher Scientific) containing 5% FCS, and gentamicin, supplemented with 30 ng/ml rmM-CSF for macrophage differentiation or 30 ng/ml rmM-CSF and 20 ng/ml rmRANKL (both, R&D Systems Minneapolis, MN, USA) for osteoclast differentiation. To study the resorption ability additional ER-Hoxb8 cells were seeded on 650 μm-thick bone slices or on a 96-well calcium phosphate–coated plate at a density of 50,000 cells/slice or per well in the osteoclast differentiation medium described above.
**Isolation, culture, and differentiation of BM precursors on plastic**

BM was isolated from the femur and tibia by flushing the marrow cavity. For differentiation into osteoclasts, total BM cells were seeded into 96-well plates at a density of 100,000 cells/well in 150 µl α-MEM medium, containing 5% FCS and gentamicin, supplemented with 30 ng/ml rmM-CSF and 20 ng/ml rmRANKL. Additional BM cells were seeded on 650-µm-thick dentin or bone slices or on 96-well calcium phosphate–coated plate at a density of 100,000 cells/well or per slice. Culture medium was refreshed every 3 d.

**TRAP staining and cell count**

Differentiated osteoclasts on tissue-cultured plates, bone, or calcium phosphate–coated plates were washed in PBS, fixed in 4% PBS-buffered formaldehyde for 10 min, and stained for TRAP activity, using the leukocyte acid phosphatase kit (Sigma-Aldrich), according to the manufacturer's protocol. Digital images were obtained with a camera mounted on an inverted light microscope (Leica Microsystems, Wetzlar, Germany).

**RNA isolation and quantitative PCR**

Total RNA of cultured cells was isolated using TRIzol reagent (Sigma-Aldrich) and reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase, oligo(dT) primers, and dNTPs (Thermo Fisher Scientific). Quantitative, real-time PCR was performed using the Step One Plus real-time PCR system and SYBR Green Master mix (both Thermo Fisher Scientific). The primer sequences are listed in Supplemental Table 1. GAPDH was used as the housekeeping gene. Expression of this gene was not affected by the experimental conditions. Samples were normalized for the expression of GAPDH by calculating the comparative threshold (Ct, cycle threshold; i.e., \( -\Delta Ct = Ct\) GAPDH \(- Ct\) gene of interest).

**Resorption assay on bone slices**

After 10 d of culture, bone slices were stained with Coomassie brilliant blue, and the percentage of resorbed area was quantified, as previously described 17.

**Resorption assay on calcium phosphate–coated plates**

Calcium phosphate–coated plates were prepared as previously described by ten Harkel et al. 18. To analyze the pit formation on calcium phosphate–coated plates, the medium was removed from the wells on d 10, and 100 µL of 10% bleach solution was added. Cells were incubated with the bleach solution for 5 min at room temperature and were washed twice with distilled water. Digital images were obtained with a camera mounted
CRISPR/Cas9 GENE EDITING OF ER-Hoxb8 OSTEOCLAST PRECURSORS AS A TOOL TO STUDY OSTEOCLAST BIOLOGY

on an inverted-light microscope. The percentage of resorbed bone area was quantified using the LAS image-analysis system (Leica).

**F-actin staining and confocal microscopy**

Actin-ring formation was analyzed using confocal microscopy, as previously described. Image stacks spanning the entire osteoclast were generated using Olympus FV1000 Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan). The number of actin rings and actin ring surfaces expressed as percentage of the osteoclast surface was assessed.

**FACS analysis**

ER-Hoxb8 CRISPR-Cas9 genetically modified clones were differentiated for 6 d toward macrophages in RPMI 1649 containing 10% FCS and gentamicin, supplemented with 15 ng/ml rmM-CSF. Undifferentiated and ER-Hoxb8 cells differentiated toward macrophages were labeled with PE/Cy7 anti-mouse F4/80 (BioLegend, San Diego, CA, USA). Fluorescence was analyzed with CyAn Flow Cytometer and Kaluza Analysis software 1.3 (Beckman Coulter, Brea, CA, USA).

**CRISPR/Cas9 plasmid and clone generation**

LentiCRISPRv2 was a gift from Feng Zhang (Addgene plasmid 52961; Addgene, Cambridge, MA, USA). gRNA sequences directed against exon 2 of the murine NFATc1 and DC-STAMP genes were obtained from the CHOPCHOP web tool for genome engineering. Analysis of likely off-target genes was performed in silico. No genes or no genes directly involved in osteoclast differentiation were identified as off-target. A gRNA targeting GFP was used as control, in addition to nontreated cells. The following CRISPR guide oligonucleotides were ordered: GFP control, gRNA (forward: 5′-CACCGGGGCGAGGAGCTGTTCACCG-3′, reverse 5′-AAACCGGTGAACAGCTCCTCGCCCC-3′), NFATc1 gRNA (forward: 5′-CACCGGTAGTTGGACTCGTAGGAGG-3′, reverse: 5′-AAACCCTCCTACGAGTCCAACCTACC-3′), and DC-STAMP gRNA (forward: 5′-CACCGGGCTCATATGAATGACACTAG-3′, reverse: 5′-AAACCTAGTGTCATTCATATGAGCC-3′) (Biolegio, Nijmegen, The Netherlands) and were cloned using the BsMBI overhangs. After sequence verification of the insert, lentivirus was made according to Broeren et al.

Virus concentrations were determined using the INNOTEST HIV antigen mAb kit (Diasorin, Saluggia, Vercelli, Italy). Lentiviral transduction was performed with 400 ng lentivirus/100,000 cells in the presence of 8 µg/ml polybrene (Sigma-Aldrich). Cells were subsequently selected with 8 µg/ml puromycin for 72 h and used for a surveyor assay or for clone generation. Genomic modification of single-cell clones was assessed with regular PCR (see “Surveyor assay” below) and sequencing.
Surveyor assay

PCR primer pairs, spanning the genomic gRNA target site of NFATC1 exon 2 and DC-STAMP exon 2, were generated (primer sequences NFATc1: forward, 5′-GTCTGACCTCAGGCCATTCC-3′, reverse, 5′-AGGTCCAGAGTGCTATCGGT-3′; DC-STAMP: forward, 5′-ATGTGTTTCCACGAAGCCCT-3′, reverse, 5′-AAGTGCTTGTGAGCCCCCTAC-3′ (Primer-Blast; National Center for Biotechnology Information, Bethesda, MD, USA). Genomic DNA was isolated from ER-HoxB8 cells (Qiagen 69504; Qiagen, Valencia, CA, USA), and PCR reactions were optimized with 200 ng genomic DNA and a high-fidelity, proof-reading polymerase (Phusion; New England BioLabs Inc., Ipswich, MA, USA). Amplicon size, yield, and background bands were evaluated using standard agarose gel electrophoresis. Subsequently, PCR amplicons derived from CRISPR/Cas9-treated cell pools were generated, purified, and concentrated (Qiagen 28004). DNA concentration was determined (nanodrop) and equalized among samples. Heteroduplex formation was performed in optimized buffer conditions with 400 ng DNA in a thermocycler (T100; Bio-Rad Laboratories, Hercules, CA, USA) according to the IDT Surveyor mutation-detection users guide (Integrated DNA Technologies, Coralville, IA, USA). Surveyor nuclease digestion was performed for 5 min at 42°C. Genomic mutations were detected on 2% agarose gels, and images were captured with a Bio-Rad Gel Doc Xr+.

Statistical analysis

Statistical differences were calculated by Student’s t test, using GraphPad Prism software (version 5.03; GraphPad Software, La Jolla, CA, USA). P < 0.05 was considered significant.

Results and discussion

ER-Hoxb8 cells differentiate into TRAP+ multinucleated cells

The central aim of our work was to generate, from C57BL/6 WT ER-Hoxb8 cells, a clonal cell line that strongly resembled the phenotype and function of primary osteoclasts and to delete specific genes using CRISPR/Cas9 technology. We first generated ER-Hoxb8 cells from the BM of C57BL/6 WT mice, as previously described11. Before proceeding with limiting dilution to obtain a clonal cell line, we investigated the ability of the ER-Hoxb8 cells to differentiate toward osteoclasts. As shown in Figure 1A, after 6 d of stimulation with osteoclastogenic factors M-CSF and RANKL, the cells differentiated toward multinucleated, TRAP+ cells. Starting from this total population with osteoclastogenic potential from limiting dilution, we obtained a clonal cell line and characterized it for efficiency of differentiation, expression markers, and functionality, comparing it with conventional BM-derived osteoclasts. ER-Hoxb8 cells were cultured in
the presence of M-CSF and RANKL on tissue-culture plates for 4 d and on calcium phosphate–coated plates and bone slices for 6 d. ER-Hoxb8 cells differentiated into TRAP+ multinucleated cells on all 3 substrates (Figure 1B, C, and D respectively). The differentiation and fusion of ER-Hoxb8 cells toward osteoclasts resemble the ones of BM cells (Figure 1E).

**Figure 1. ER-Hoxb8 cells differentiate into multinucleated TRAP+ osteoclasts.**

(A) TRAP staining of the total population of ER-Hoxb8 cells differentiated for 6 d on plastic plates. TRAP staining of single-clone ER-Hoxb8 cells and BM osteoclasts differentiated for 4 d on plastic plates (B) or for 6 d on bone slices (C) and calcium phosphate–coated plates (D). (E) The number of ER-Hoxb8–derived osteoclasts (gray bars) and BM-derived osteoclasts (black bars) after 4 d of differentiation on plastic plates. Values are the means ± SD of 3 experiments.

**ER-Hoxb8–derived osteoclasts show strong up-regulation of osteoclast markers**

To investigate the differentiation of the clonal Hoxb8 cell line in greater detail, gene expression of various osteoclast and macrophage markers was measured in 4-d, differentiated, ER-Hoxb8–derived osteoclasts and compared with expression in undifferentiated ER-Hoxb8 cells and ER-Hoxb8–derived macrophages. Figure 2A depicts a schematic representation of an osteoclast with the various markers measured. Figure 2B shows the mRNA levels of receptors for key osteoclast differentiation factors (c-Fms and RANK) and various osteoclast markers. c-Fos and NFATc1 are key transcription factors for activation of osteoclastogenic differentiation, whereas DC-STAMP is involved in the fusion of single, mononucleated cells into resorbing, multinucleated osteoclasts. TRAP is a lysosomal enzyme strongly expressed by osteoclasts, whereas αVβ3 integrin is the major adhesion receptor on osteoclasts. Carbonic anhydrase II and CIC-7 are necessary for the acidification of the resorption lacunae, while cathepsin K and MMP-9 are the major enzymes secreted into resorption lacunae to degrade the organic part of bone. CTR is expressed on mature osteoclasts and is implicated in the regulation of
osteoclast function by binding calcitonin. RANK and c-Fms were significantly up-regulated after 4 d of differentiation with M-CSF and RANKL, compared with undifferentiated ER-Hoxb8 cells. All the other markers were also significantly up-regulated in osteoclasts both compared with undifferentiated and with macrophage differentiated ER-Hoxb8. Finally, CTR also showed strong up-regulation compared with undifferentiated and macrophages ER-Hoxb8 cells, although statistical analysis could not be performed because this gene was not detectable in the other 2 cell types. In contrast, the macrophage marker F4/80 was down-regulated in ER-Hoxb8–derived osteoclasts compared with ER-Hoxb8–derived macrophages. Taken together, these data suggest that ER-Hoxb8 is able to differentiate into osteoclasts. Moreover, our results show that the differentiation of ER-Hoxb8 cells into osteoclasts is specifically induced by osteoclastogenic differentiation factors (RANKL plus M-CSF) and not by the removal of β-estradiol from the culture medium.

**ER-Hoxb8– and BM-derived osteoclasts display comparable kinetics and expression levels of osteoclast markers**

To investigate the process of differentiation of ER-Hoxb8 cells toward osteoclasts in more detail, the expression of c-Fms, RANK, and osteoclast markers (c-Fos, NFATc1, DC-STAMP, αVβ3 integrin, TRAP, carbonic anhydrase II, CIC-7, cathepsin K, MMP-9, and CTR) were monitored in ER-Hoxb8 cells and BM cells stimulated with M-CSF and RANKL over time (from d 0 until d 6 of differentiation) (Figure 3). Despite differences in their initial expression levels, as expected because of the heterogeneity of cells present in BM, at later time points, the kinetics and levels of expression for all genes evaluated were strongly comparable between ER-Hoxb8 and BM-derived osteoclasts. In general, the stimulation with M-CSF and RANKL resulted in a time-dependent, increased expression of c-Fms, RANK, and other osteoclast differentiation markers. In both cell types, c-Fms and RANK were strongly up-regulated by the first days of differentiation, which is according to their key role in the starting step of differentiation. NFATc1 and c-Fos reached their highest expression around d 3, whereas the other osteoclast marker genes (DC-STAMP, TRAP, cathepsin K, CIC-7, carbonic anhydrase II, and MMP-9) peaked around d 4 both in ER-Hoxb8 and BM-derived osteoclasts. The levels of gene expression of osteoclast markers correlated well with the morphologic changes observed during the differentiation in the cell culture in which, at d 4, numerous multinucleated osteoclasts were visible. The kinetics of CTR expression was slower, and the highest expression was reached around d 6 for both cell types. These results show that the differentiation process of ER-Hoxb8 cells toward osteoclasts closely resembles the process of BM precursors.
Figure 2. ER-Hoxb8 osteoclast strongly up-regulate osteoclast markers.

(A) Schematic representation of an osteoclast, clarifying the functions of the diverse proteins. (B) ER-Hoxb8 cell mRNA expression before (gray bars) and after 4 d of differentiation in presence of RANKL and M-CSF (black bars) or only M-CSF (white bars). Expression of macrophage marker F4/80, receptors for differentiation factors RANK and c-Fms, and osteoclast marker NFATc1, c-Fos, DC-STAMP, integrin β3, TRAP, CIC-7, carbonic anhydrase II, MMP-9, cathepsin K, and calcitonin receptor was assessed. Gene expression was normalized for GAPDH and expressed as −ΔCt. *P < 0.05, **P < 0.01 for ER-Hoxb8-derived osteoclasts vs. ER-Hoxb8 undifferentiated cells, and +P < 0.05, ++P < 0.01 for ER-Hoxb8-derived osteoclasts vs. ER-Hoxb8-derived macrophages, by Student’s t test. The means ± SD of 3 experiments performed in triplicate are shown.
Figure 3. ER-Hoxb8– and BM-derived osteoclasts show comparable kinetics and expression levels for osteoclast markers. Kinetic profiles of gene expression by ER-Hoxb8 cells (gray) and BM cells (black) during differentiation toward osteoclasts. Expression of RANK, c-Fms, c-Fos, NFATc1, DC-STAMP, integrin β3, TRAP, carbonic anhydrase II, CIC-7, cathepsin K, MMP-9, and CTR was assessed from d 0 until d 6 of culturing with M-CSF and RANKL. Expression was normalized for GAPDH, and values are expressed as $-\Delta Ct$. Means ± SD of 5 BM and ER-Hoxb8 cell differentiations are shown.

**ER-Hoxb8–derived osteoclasts are able to form actin-rings and to resorb bone**

After attachment to bone, osteoclasts exhibit differentiation and polarization; this process includes the organization of F-actin cytoskeleton into a typical, densely packed, ring-like structure (an actin-ring) that seals the area beneath the cell and forms a closed compartment in which bone can be resorbed through secretion of acid compounds and
enzymes. An actin-ring formation is, thus, a prerequisite for the resorbing activity of osteoclasts. The presence of an actin-ring was evaluated through fluorescent staining Alexa Fluor 488–conjugated phalloidin and confocal microscopy. Similar to BM-derived osteoclasts ER-Hoxb8–derived osteoclasts formed actin-rings (Figure 4A). The number of actin-rings and the percentage of ring area per osteoclast were quantified in ER-Hoxb8– and BM-derived osteoclasts after 8 d of differentiation on bone slices (Figure 4B).

Next, we evaluated the functionality of ER-Hoxb8–derived osteoclasts on bone resorption. ER-Hoxb8 and BM cells were seeded on bone slices and on calcium phosphate–coated plate and cultured for 10 d in the presence of M-CSF and RANKL. Coomassie blue staining of bone slices as well as calcium phosphate–coated plate showed the presence of resorption pits created by ER-Hoxb8–derived osteoclasts on both materials (Figure 4C and D). The area that was eroded by Hoxb8 osteoclasts after 10 d of culture on the calcium phosphate–coated plate was measured and compared with BM osteoclasts (Figure 4E). Although the area eroded by non-stimulated ER-Hoxb8 cells was less compared with non-stimulated BM-derived osteoclasts, LPS stimulation (1 µg/ml) clearly stimulated the resorptive activity of both cell types (ER-Hoxb8: mean fold increase 1.4× compared with non-stimulated cells, determined in 3 independent experiments; BM-derived osteoclasts: mean fold increase 1.6×, determined in 2 independent experiments). These data indicate that ER-Hoxb8–derived osteoclasts show strong resemblance to primary osteoclasts not only in phenotype but also in functionality.

**ER-Hoxb8 can be genetically modified using CRISPR/Cas9 technology**

To assess the ER-Hoxb8 osteoclast system for its suitability in fundamental osteoclast research we used the CRISPR/Cas9 technology to inactivate specific genes in this cell line. To validate our protocol, we focused on inactivation of NFATc1, the master transcription factor for osteoclast differentiation and DC-STAMP, crucial in the process of the fusion of mononucleated osteoclasts into multinucleated, mature osteoclasts. $^{22-25}$ gRNA sequences targeting the murine NFATc1 or DC-STAMP genes were inserted in a lentiviral expression vector (Figure 5A). ER-Hoxb8 cells were transduced and selected to enrich for CRISPR/Cas9-expressing cells. Genomic modification with CRISPR/Cas9 leads to specific deletions at or near the gRNA sequence in the NFATc1 or DC-STAMP genes, which can be detected with a surveyor nuclease assay in heterogeneous cell populations. The efficiency of gRNAs to induce genomic mutations was assessed with a surveyor nuclease assay. Surveyor nuclease is an enzyme that cuts a DNA heteroduplex if it contains mismatches. Without CRISPR/Cas9 activity, such heteroduplexes are absent, and only a single-PCR amplicon is detected. As shown in Figure 5B, a substantial
percentage of CRISPR/Cas9–treated cells have a genetic modification at the expected
target sequence of NFATc1 (top panel) and DC-STAMP (lower panel). The NFATc1
amplicon is 1 kb long and is present in the DNA of the WT control; 2 smaller bands (594
bp and 406 bp) are visible in CRISPR/Cas9-treated cells. That size corresponds to the
expected Cas9-induced cut site directed by the gRNA. The DC-STAMP amplicon is 997 bp
long and is present in the WT control; a single, smaller band is visible in CRISPR/Cas9-
treated cells. The expected Cas9 cut site was almost exactly in the middle of the
amplicon (514 bp and 483 bp); therefore, only 1 CRISPR/Cas9-induced band was visible.

Figure 4. ER-Hoxb8 osteoclasts can form actin-ring and are able to degrade
bone and calcium phosphate–coated plates. (A) Examples of ER-Hoxb8– and BM-derived
osteoclasts forming actin rings after 8 d of culture on bone
slices. Surface marker CD44
was stained using the rat anti-
CD44 Ab and was visualized
with Alexa Fluor 647–
conjugated goat anti-rat IgG
(blue); nuclei were stained
with DAPI (red), and actin was
stained with Alexa Fluor 488 –
conjugated phalloidin (green).
Images were generated with a
Olympus FV1000 confocal
laser scanning microscope. (B)
The number of actin rings per
osteoclast and the percentage
of actin-ring area per
osteoclast in ER-Hoxb8– and
BM-derived osteoclasts.
Horizontal and vertical line
show the means ± SD (ER-
Hoxb8, n= 39 osteoclasts; BM,
n = 26 osteoclasts). Pictures of
resorption pits on bone slices
were visualized with
Coomassie brilliant blue
staining (C) and on calcium
phosphate–coated plates (D)
after 10 d of ER-Hoxb8 and BM
culture. (E) Quantification of
the calcium phosphate–eroded area at d 10 is expressed as a percentage. Values are the means ± SD of 3
experiments for ER-Hoxb8 and 4 experiments for BM. *P <0.05, **P <0.01 by Student’s t test.
The Hoxb8 cells obtained were expanded, which was followed by limiting dilution to obtain single clones with a homozygous genetic modification. DNA was isolated from the single cell clones, and genomic PCR amplification, followed by DNA sequencing of the target locus, was performed to identify clones that exhibited a biallelic mutation. Multiple mutated clones were obtained. Table 1 shows a table with an overview of the clones obtained. For NFATc1, we obtained 2 clones carrying homozygous mutations; clone 1 had a deletion of 187 bp, and clone 2 had one of 53 bp. For DC-STAMP, we obtained 2 clones with smaller homozygous mutations; clone 1 showed a deletion of 26 bp, and clone 2 had a deletion of 9 bp preceded by a mutation of 7 bp (Figure 5C). To assess whether the lentiviral transduction and expression of the CRISPR/Cas9 construct influenced the osteoclastogenic differentiation of ER-Hoxb8 cells, the same procedure was applied to generate single-cell clones of ER-Hoxb8 cells transduced with a gRNA that had no genomic target (directed against GFP). Two of the generated clones were used as controls in the following differentiation experiments.

**ER-Hoxb8 cells with homozygous deletions in NFATc1 or DC-STAMP genes lost their ability to differentiate toward mature osteoclasts at the expected differentiation stages**

Finally, we evaluated the differentiation of NFATc1- and DC-STAMP-mutated ER-Hoxb8 cells toward osteoclasts. ER-Hoxb8 clones were expanded and stimulated with RANKL and M-CSF for 6 d. As shown in Figure 6A and D, ER-Hoxb8 cell clones treated with a gRNA targeting GFP efficiently differentiated into mature TRAP+ osteoclasts with strong fusion of cells with ≥10 nuclei. In contrast, functional impairment of NFATc1 in ER-Hoxb8 cells showed complete inhibition of differentiation toward osteoclasts; no TRAP+ cells were present after the differentiation process (Figure 6B). This is similar to an early block of differentiation into osteoclasts when the NFATc1 protein is knocked out. In contrast, functional impairment of DC-STAMP led to the formation of mononucleated TRAP+ cells, but fusion into multinucleated mature osteoclasts was impaired (Figure 6C and D), similar to the described function of DC-STAMP. Moreover, targeting ER-Hoxb8 cells with CRISPR/Cas9 did not affect differentiation of ER-Hoxb8 cells into macrophages, as shown by the increased expression of the macrophages marker F4/80 by all genetically modify ER-Hoxb8 cells upon M-CSF stimulation. In Figure 6E, representative graphs of FACS analysis of one clone/gRNA are shown.
Figure 5. ER-Hoxb8 cells can be efficiently modified genetically with CRISPR/Cas9 technology and lentivirus transduction. (A) Schematic representation of the RNA-guided Cas9 nuclease targeted to the second exon of NFATc1 and DC-STAMP genes. The gRNA sequence (red) pairs with the DNA target (black), directly upstream of the adjacent motif (PAM sequence, blue). Cas9 cleaves the dsDNA 3 bp upstream of the PAM. (B) Gel from the Surveyor Assay proved the presence of the genetic modification in the heterogeneous population of cells transduced with lentivirus containing CRISPR/Cas9 gRNA against NFATc1 or DC-STAMP. (C) DNA sequencing of WT and the 2 mutated clones confirmed the presence of genomic mutations.
Cells transduced with lentivirus containing CRISPR/Cas9 gRNA against NFATc1 or DC-STAMP

In conclusion, our results indicate that ER-Hoxb8 cells represent a valuable and efficient tool for studying osteoclasts in vitro, reducing the time-consuming procedure of BM isolation and the number of animal used for in vitro experimentation. The features of this cell line allow its use in the study of different aspects of osteoclast biology. First, the conditional immortalization represents a big advantage, that is the expression of Hoxb8 is switched off during the differentiation and in mature osteoclasts, preventing possible side effects. Second, the homogeneity of the starting population and the comparable
kinetics of differentiation with BM-derived osteoclasts make these cells a useful tool for deep investigation of the differentiation process toward osteoclasts. Third, actin-ring formation and efficient bone degradation allow the use of this cell line for functional assays. The possibility to inactivate genes specifically by biallelic mutation in ER-Hoxb8 myeloid precursors with CRISPR/Cas9 technology represents a markedly easy, highly-specific, and efficient method of generating a potentially unlimited number of genetically modified precursors, allowing functional studies of many known and unknown proteins involved in osteoclast differentiation and function.

Acknowledgments

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References

Supplemental Table 1

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CHAPTER 8

Summary and final considerations
Summary & Discussion

In this thesis we investigated the impact of innate immunity on joint pathology during rheumatoid arthritis (RA). Innate immunity is activated in diseases that have been described to coincide with the development of joint destruction in RA. Periodontal disease (PD) and metabolic syndrome (MetS) are two important examples of such comorbidities of joint destruction in RA. Since persistent activation of the innate immune system is the common thread throughout these inflammatory comorbidities, a deeper understanding of the differential mechanisms underlying this chronic hyperactivation will help to develop more effective treatments. Overall, in this thesis we investigated the effects of various factors on the activation of innate immune cells, such as macrophages, osteoclasts, and their precursors and their influence on the development of RA. In particular, we zoomed in on the effects of the pro-inflammatory cytokine interleukin (IL)-1 and the alarmin S100A8/A9, as well as on the role of Fc gamma receptors (FcγRs) and systemic factors like low density lipoprotein cholesterol (LDL-C) and oxidized LDL-C (oxLDL) that promote the activation of innate immunity, thus influencing joint destruction.

In Chapter 2 we sought to get a better understanding of the link between enhanced IL-1 signaling and the susceptibility to develop RA. Erosion of bone is largely mediated by osteoclasts that differentiate from osteoclast precursor populations (OCPs) residing within the bone marrow. Using IL-1 receptor antagonist knockout (Il1rn−/−) mice we explored the effects of enhanced IL-1 signaling on the composition of three different OCP populations at four different bone locations (long bone, calvaria, vertebra, and jaw), and its effects on in vitro osteoclastogenesis and bone resorption. As dysregulation of IL-1 signaling resulted in the expansion of OCP subsets in the BM of long bone and jaw, this can increase osteoclastogenesis at those skeletal sites (Figure 1) and lead to enhanced activation of innate immunity pathways involved in bone destruction. Interestingly, osteoclast cultures from all the various bone locations showed increased in vitro bone resorption underlining that differences in the resorptive activity observed in vivo under inflammatory conditions may be also related to differences in the type of bones. Together, these findings suggest that IL-1-driven OCP expansion in long bone and jaw can represent a mechanistic link underlying bone destruction in RA and PD pathology.
Another factor that is crucial to the activation of the innate immune system is the interaction between Fcγ receptors (FcγRs) present on innate immune cells and immunoglobulin G (IgG) containing immune-complexes (ICs) that accumulate in the synovium of seropositive RA patients. Alterations in the expression of FcγRs are often found in the synovial tissue of RA patients \(^1\)-\(^3\), pointing out a role for them in RA etiopathogenesis. Furthermore, the pathogenic role of individual receptors was investigated in various experimental models of arthritis \(^4\)-\(^6\). However, the complete absence of all FcγRs and the particular role of FcγRIV to the development of bone destruction and the underlying mechanisms remains to be elucidated. Therefore, in Chapter 3 we studied IC-mediated antigen-induced arthritis (AIA) in mice that lack all FcγRs and compared the development of bone destruction between FcγRI,II,III,IV deficient (FcγRI,II,III,IV\(^{-/-}\)) and their wild-type (WT) controls. Complete absence of FcγRs lowered bone erosion and osteoclast activity as compared to WT mice. This was a result of decreased joint inflammation, even though both the humoral and cellular immune responses were not impaired. Of note, when we compared the development of arthritis between FcγRI,II,III\(^{-/-}\) mice and their WT controls we found that in contrast to
FcγRI,II,III,IV^-/-, FcγRI,II,III^-/- have increased bone erosion, inflammation, and numbers of neutrophils. Interestingly, the additional absence of FcγRIV mainly decreased the numbers of neutrophils present in the joint, suggesting a crucial role for this FcγR in the recruitment of neutrophils during experimental arthritis. Moreover, bone erosion significantly correlated with the number of neutrophils as well as that of S100A8-positive cells present in the joint. Together, these findings suggest that activating FcγRs play a crucial role in the development of bone erosion during AIA by inducing inflammation, and that FcγRIV mediates bone erosion in AIA by stimulating the influx of S100A8/A9-producing neutrophils into the arthritic joint (Figure 2). Moreover, this suggests that the targeting of the FcγRs could be promising in dampening IC-driven bone destruction in RA patients.

![Figure 2. Schematic overview of the mechanism proposed underlying the role of FcγRIV aggravates bone destruction.](image)

RA patients often suffer from dyslipidemia, characterized by high levels of LDL-C and low high density lipoprotein cholesterol (HDL-C) \(^7,8\). Whereas it is commonly accepted that LDL hypercholesterolemia increases the risk of cardiovascular diseases (CVDs) in RA patients, it is still a matter of debate whether a high level of LDL-C on itself aggravates arthritis. Of interest, RA patients with low LDL-C levels show increased disease activity and a higher risk to develop CVDs, resulting in what is known as ‘lipid paradox’ \(^9,10\). In an
inflamed joint, high levels of LDL-C are rapidly oxidized to oxLDL, which is primarily taken up by macrophages. The accumulation of oxLDL triggers the production of many pro-inflammatory as well as anti-inflammatory factors, thereby modulating the activity of innate immune cells. Since this could have an impact on the development of arthritis, in Chapter 4 and Chapter 5, we investigated the effect of high levels of LDL-C/oxLDL on synovial inflammation and joint destruction during AIA using hypercholesterolemic apolipoprotein E-deficient (Apoe<sup>−/−</sup>) mice. In Chapter 4, we observed reduced inflammation and cartilage damage in Apoe<sup>−/−</sup> mice after induction of AIA. In contrast, high LDL-C levels present in naïve Apoe<sup>−/−</sup> mice had no effect on either synovial inflammation or cartilage destruction, suggesting that oxLDL formation in the inflamed joint, but not high LDL-C levels by itself, affect the onset and progression of joint inflammation and cartilage destruction in AIA. Given the crucial role of macrophage FcγRs during IC-mediated AIA, we investigated in vitro the effects of high LDL-C/oxLDL on the expression of FcγRs on macrophages. As we found that oxLDL, but not LDL-C lowered the expression of activating receptors this suggests that high LDL-C levels, and their increased oxidation in the inflamed joint, suppress inflammation and cartilage destruction by regulating macrophage FcγR expression (Figure 3). In Chapter 5, we investigated more in detail the effect of high LDL-C/oxLDL levels on osteoclastogenesis and bone destruction. Induction of AIA resulted in a strong reduction of bone destruction in Apoe<sup>−/−</sup> mice as compared to WT controls and was accompanied by a reduction of osteoclast numbers on the cortical bone. Whereas the absence of Apoe did lower neither the numbers of OCPs in the BM nor their in vitro osteoclastogenesis, exposure of pre-osteoclasts and osteoclasts to oxLDL, but not LDL-C strongly inhibited osteoclast formation and their resorptive activity (Figure 3). Interestingly, this coincided with a sharp reduction of osteoclast-associated receptor (Oscar) and the DNAX adaptor protein-12 encoding gene Tyrobp of the immunoreceptor tyrosine-based activation motif (ITAM) co-stimulation pathway that is strongly involved in osteoclastogenesis. Together, these findings suggest that in the inflamed joint, high LDL-C levels lessen bone destruction during AIA, probably due to the formation of oxLDL that inhibits osteoclast formation and activity through modulation of the ITAM-signaling.
Figure 3. Schematic overview of the hypothesized mechanisms of oxLDL-driven reduction of RA pathology. Immune complexes (ICs) in the inflamed synovium increase the macrophage expression of activating FcγRs, resulting in enhanced production of matrix metalloproteinases (MMPs) that stimulate the degradation of the cartilage extracellular matrix (ECM), but also pro-inflammatory cytokines that can enhance bone destruction. In contrast, synovial inflammation in combination with hypercholesterolemia leads to enhanced LDL-C oxidation. Oxidized LDL-C (oxLDL) decreased the FcγR expression on macrophages, thereby leading to decreased MMP activity and cartilage damage. Furthermore, this may reduce the secretion of pro-inflammatory cytokines/chemokines, thereby impairing the ability of macrophages to promote osteoclastogenesis. Finally, oxLDL directly inhibits osteoclast differentiation by down-regulating the ITAM-pathway, thus resulting in decreased bone destruction during experimental arthritis.

Next to a major role in regulating lipid transportation and accumulation, APOE is also involved in several immunological processes such as suppression of T cell proliferation and neutrophil activation, regulation of macrophage functions, and modulating inflammation and lipid oxidation. Moreover, previous studies have shown that different allelic variants of human ApoE (APOE-ε2, APOE-ε3, and APOE-ε4) are associated with the severity of various inflammatory diseases such as atherosclerosis and Alzheimer’s disease in which inflammation and activation of the innate immune system play an important role. Association studies generated conflicting results with respect to the various Apoe genotypes and RA development. A previous study found that the APOE-ε4 genotype was less frequent in RA patients compared to healthy subjects compared to the other two genotypes, suggesting a protective role for APOE4 in RA. Other studies showed that APOE4 is associated with more severe dyslipidemia and as such can influence the innate immune response and thus the development of
SUMMARY AND FINAL CONSIDERATIONS

RA. As we find that LDL hypercholesterolemia decreases joint pathology in experimental RA, it can be that subjects with the APOE-ε4 genotype are protected against the development of RA probably because of the suppressive effect of oxLDL present in the arthritic joint. Whereas the involvement of APOE genotypes with respect to the risk of developing RA has been investigated to some extent, very little is known about their influence on other rheumatic diseases where innate immunity plays a likewise important role such as osteoarthritis (OA). Therefore, in Chapter 6 we studied the effect of APOE polymorphisms (APOE-ε3 and APOE-ε4) on OA pathology. In this study, we induced experimental OA using targeted replacement mice, where the mouse Apoe gene was replaced by the human isoform. We found that APOE-ε4 mice developed more severe inflammation, as well as cartilage destruction and ectopic bone formation as compared to APOE-ε3 mice, suggesting that APOE genotypes may represent a risk factor for OA.

Bone destruction is a major feature of RA as the result of increased numbers and activity of osteoclasts as is underlined in previous chapters. Currently, in vitro studies on mouse osteoclasts are mainly performed on BM-derived cells with a negative impact on the number of animals used and the limited number of cells available as well as the heterogeneity of the source. Therefore, in Chapter 7 we validated the use of the ER-Hoxb8 cell line of conditionally immortalized monocyte/macrophage mouse progenitor cells for the differentiation into functional osteoclasts. In this study, from C57BL/6 BM cells we produced an ER-Hoxb8 clonal cell line that strongly resembles both phenotype and function of BM-derived osteoclasts. Then, CRISPR/Cas9 technology was applied to show that this model can be used to specifically inactivate genes by biallelic mutation. More specifically, we generated Nfatc1−/− and Dcstamp−/− ER-Hoxb8 cells that lack the ability to differentiate into osteoclasts or to fuse into multinucleated osteoclasts, respectively. In conclusion, this method represents an efficient and highly specific system to generate potentially unlimited numbers of genetically modified OCPs for in vitro study of osteoclast behavior under both physiological and inflammatory conditions.

Future perspectives & final considerations

Conventional therapies for RA mainly include the use of corticosteroids and disease-modifying anti-rheumatic drugs (DMARDs), in particular, methotrexate. However, biological drugs have demonstrated a better efficacy at reducing the pathological effects of autoreactive B and T cells as well as those of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 that are crucial in driving RA.
Anti-TNF-α treatment is currently the most frequently used biotherapy for RA patients. However, those patients with severe destruction who do not respond to TNF-α blockers could benefit from anti-IL-1 therapies. Several clinical studies have shown the beneficial effects of pharmacological inhibition of IL-1 activity in chronic inflammatory diseases. Approved IL-1 therapeutics aim to target the IL-1-receptor (e.g.: Anakinra: the recombinant human interleukin-1 receptor antagonist (IL-1Ra)) or neutralize IL-1 activity (e.g.: Canakinumab: IL-1β-neutralizing antibody, Rilonacept: soluble IL-1 decoy receptor). Among them Anakinra is currently the most used anti-IL-1 therapy due to its efficacy at reducing clinical symptoms, inflammation, and radiographic progression, but also owing to its safety, short half-life and multiple routes of administration. In line with this, administration of IL1Ra reduced pathology in various experimental models of arthritis. As we find that the absence of IL-1Ra increased the numbers of OCPs in long bone and jaw, we assume that dysregulation of IL-1 signaling would result in a larger reservoir of precursors available to differentiate into osteoclasts under inflammatory conditions. Therefore, the targeting of IL-1-signaling might be particularly beneficial in the treatment of a specific subgroup of RA patients with severe bone destruction and PD patients with alveolar bone loss that do not respond to anti-TNF-α therapies. Anti-IL-1 therapy significantly reduces bone destruction in both RA and PD and has been suggested to be also effective in treating RA patients with comorbidities.

Given the importance of the IC-FcγRs signaling in the development of RA pathology, its targeting would represent a valid alternative therapy for those patients who do not respond to conventional and biological therapies. Despite the importance of IC-FcγRs signaling in joint pathology, which has been extensively proven in many experimental models of RA, it is difficult to directly translate these results to the human situation as FcγRs present in mice do not completely overlap with those in humans. Thus, further investigations are needed to elucidate the exact role and mechanisms of action of the single FcγRs during different phases of disease. Particularly, little is known about the contribution of the murine FcγRIV, and its human ortholog form FcγRIIIA, to the development of RA. Selective depletion of macrophages in RA synovium as well as in experimental arthritis has been shown to reduce inflammation and pathology. Activating FcγRs on synovial macrophages modulate bone destruction by triggering the production of pro-inflammatory mediators in various myeloid cells, resulting in higher production of pro-inflammatory cytokines and accumulation of ICs, but can also directly lead to the activation of osteoclasts. Particularly, the expression of FcγRI (also known as CD64) is strongly induced by pro-inflammatory cytokines on both M1- and M2-macrophages. FcγRI has a high affinity for monomeric IgGs, therefore plays a crucial role in initiating and fostering immune response even at low IgG concentrations. This makes CD64 an attractive candidate target for immunotherapy. Of interest, the selective targeting of pro-inflammatory M1-macrophages with anti-CD64 immunotoxins...
has been recently proposed as a promising therapeutic approach without affecting the reparative and anti-inflammatory capacities of M2-macrophages. However, most strategies make use of bacteria or plant-derived toxins that can be immunogenic and to date lack approval for clinical use in chronic inflammatory diseases. Thus, further in vitro and in vivo studies including transgenic mouse models expressing human CD64 and RA synovium graft in immunodeficient mice would be of great help in developing more specific CD64-directed antibodies to test its therapeutic potential. Our findings in the IC-driven AIA model of arthritis suggest that FcγRIV, the murine ortholog of the human FcγRIIIA, can mediate bone erosion via modulation of the influx of neutrophils producing S100A8/A9 into the arthritic joint. Interestingly, a polymorphism of FcγRIIIA results in an increased affinity of the receptor for IgG1 and IgG3 antibodies and has been associated to increased susceptibility to RA. A previous study reported that IgG stimulation of natural killer (NK) cells in subjects carrying this polymorphism results in higher Ca\(^{2+}\) influx, which may increase the formation and release of the heterodimer S100A8/A9. These alarmins are strongly increased in RA synovium and correlate with inflammation and joint destruction in various experimental models. Moreover, they enhance the production of pro-inflammatory mediators and the expression of activating FcγRs (particularly FcγRI and IV) in various immune-cells as well as osteoclastogenesis both in vitro and in vivo. Together, these observations suggest an interplay between IC-FcγRs signaling and S100A8/A9 and that targeting S100A8/A9 can be a good additional strategy to indirectly modulate the IC-FcγRs signaling, hence bone destruction during RA.

Apart from massive production of ICs and pro-inflammatory cytokines, dyslipidemia, characterized by increased levels of total cholesterol (TC) and (LDL-C), is often present in RA patients. Different phases of disease coincide with fluctuations of TC levels, with early RA being hallmarked by higher levels of TC and chronic RA by their strong reduction. LDL hypercholesterolemia significantly increases the risk of CV diseases in RA patients probably due to increased systemic LDL oxidation to oxLDL that triggers immune responses in the CV system. However, whether a high level of LDL-C on itself potentiates inflammation and joint destruction is still unclear. In fact, whereas the use of statins is beneficial in managing the cardiovascular (CV) risk in RA patients, several studies have described the risk of developing RA during statin treatment in subjects prone to develop RA. Our studies showed that not only does LDL hypercholesterolemia by itself not have an impact on RA development, but even suggest that it reduces the onset of inflammation and development of joint destruction, likely due to enhanced oxLDL formation in the arthritic environment. This probably arises from the down-regulation of FcγRs, thereby interfering with the IC-FcγR interaction needed for sustaining inflammation as described above. This hints that a blanket use of lipid-lowering drugs like statins may not always be recommendable in RA patients as this can
interfere with the oxLDL-driven suppressive effect on both cartilage and bone destruction. Interestingly, oxLDL stimulation of macrophages reduced the expression of pro-inflammatory genes in response to LPS, suggesting that prolonged uptake of oxLDL may down-modulate the signaling of innate immune pathways to counteract the inflammatory response. An earlier study showed that intra-articular injection of oxLDL resulted in increased ectopic bone formation in an experimental model of OA, likely due to the enhanced production of transforming growth factor-beta (TGF-β) by synovial macrophages. Therefore, as the elevated levels of LDL-C and their increased oxidation in the inflamed joint can increase the levels of TGF-β it may be that this promotes macrophage polarization towards the anti-inflammatory M2-like phenotype, thus contributing to resolve inflammation. Further, TGF-β strongly inhibits osteoclast formation and activity and as such can directly diminish bone destruction. This highlights that further studies are needed to shed more light on the dual character of oxLDL and its involvement during RA. Additional experiments including injections of LDL-C/oxLDL in the arthritic synovium of mice, as well as the use of inhibitors of LDL-C uptake such as proprotein convertase subtilisin/kexin type 9 (PCSK9) would help to better understand the molecular mechanisms underlying oxLDL effects.

Moreover, as we find that oxLDL inhibits osteoclast formation and activity via down-regulation of OSCAR and DAP-12 that are involved in the costimulation pathway, this suggests that the ITAM-signaling may be a valid therapeutic target to treat bone destruction in RA as alternative to the inhibition of RANK/RANKL signaling. Particularly those patients with severe bone destruction who suffer from the side effects of a prolonged use RANK inhibitors (Denosumab) could benefit from the development of such alternative treatment. Similarly to RANKL, the expression of OSCAR is strongly upregulated in RA patients and associates with disease activity. In line with this, a soluble inhibitor of OSCAR, which acts as decoy receptor, has been shown to decrease osteoclastogenesis from peripheral blood monocyte cells (PBMCs). Polymorphisms in the OSCAR promoter have been associated with low bone mineral density in postmenopausal women, further corroborating its importance in the regulation of bone metabolism. Together, previous and our findings suggest that inhibition of OSCAR is a promising therapeutic approach to decrease osteoclast activation and bone resorption in RA. Further basic and clinic research in this respect will give insights in the efficacy and long-term effects of therapies targeting the ITAM-signaling.

As enhanced osteoclast activation and bone destruction are central features of RA, a model system in which unlimited numbers of OCPs are available to conduct mechanistic studies would be a promising tool to gain a better understanding of osteoclast modulation under inflammatory conditions. Therefore, in vitro studies using the ER-Hoxb8 model system that we described in this thesis will be of great support to enable
this kind of \textit{in vivo} studies. For this model system, immortalized OCPs can be obtained from genetically modified animals allowing to study the role of various genes in osteoclast pathophysiology or genetically modified cells can be generated using CRISPR/Cas9. Moreover, the possibility to test osteoclasts from different types of bones would be an attractive tool to study site-specific osteoclast heterogeneity.

In conclusion, the findings presented in this thesis give new insights into how various triggers activate the innate immune system, thus contributing to joint destruction during RA. Moreover, this thesis highlights that particularly the development of immunotherapies targeting the activating FcγRs and a better understanding of the clinical effects of high LDL-C will lead to more effective therapies to treat RA patients who do not respond to current therapies.
References


SUMMARY AND FINAL CONSIDERATIONS


Samenvatting
Sintesi
List of Publications
Acknowledgements
RIMLS portfolio
Curriculum Vitae
Samenvatting
Reumatoïde artritis (RA) is een chronische auto-immuun ontstekingsziekte van gewrichten die 0,5-1% van de wereldbevolking treft. Voortdurende ontstekingen op zowel systemisch als lokaal niveau in de gewrichten, veroorzaakt door chronische activering van het immuunsysteem, leiden tot een progressieve schade aan kraakbeen en botten en uiteindelijk tot gewrichtsstoornissen. Hoewel bekend is dat veel factoren bijdragen aan de ontwikkeling van de ziekte, is de exacte oorzaak niet helemaal bekend. Granulocyten, monocyten, macrofagen, osteoclasten en hun voorlopers zijn allemaal cellen van het aangeboren immuunsysteem die een centrale rol spelen in de gewrichtsvernietiging veroorzaakt door ontsteking. Verder onderzoek zou daarom kunnen helpen bij het beter in kaart brengen van nieuwe mechanismen en factoren die een rol spelen bij de ontwikkeling van de ziekte. Dit zou kunnen leiden tot de ontdekking van nieuwe therapieën die zijn ontworpen om de ontwikkeling van de aangeboren immuunrespons te verstoren. Hierdoor kan het ontstaan van een vicieuze cirkel worden voorkomen en kan de ziekte vroegtijdig worden behandeld, wat de levensomstandigheden van patiënten aanzienlijk kan verbeteren.

Het doel van dit proefschrift
In dit proefschrift hebben we de impact onderzocht van aangeboren immuniteit op gewrichtsvernietiging tijdens RA. Parodontitis en het metabool syndroom zijn twee belangrijke voorbeelden van ziektes die tegelijkertijd met RA voorkomen, zogenaamde comorbiditeiten, en alledaag het gevolg zijn van de chronische activering van de aangeboren immuunrespons. Aangezien aanhoudende activering van het aangeboren immuunsysteem de rode draad is van deze inflammatoire comorbiditeiten, zou een dieper begrip van de verschillende mechanismen die aan deze chronische hyperactivatie ten grondslag liggen, helpen om effectievere behandelingen te ontwikkelen. In het algemeen hebben we in dit proefschrift de effecten van verschillende factoren op de activatie van aangeboren immuuncellen zoals macrofagen, osteoclasten en hun voorlopers bestudeerd, en als gevolg daarvan hun invloed op de ontwikkeling van RA. In het bijzonder hebben we de pro-ontstekings effecten van interleukine (IL)-1 en de alarmine S100A8/A9 onderzocht, evenals de rol van Fc-gamma-receptoren (Fcy). Al deze factoren veroorzaken ontsteking. Verder hebben we meer systemische factoren, zoals LDL-cholesterol en geoxideerd LDL-cholesterol (oxLDL) bestudeerd die de activering van aangeboren immuniteit bevorderen, waardoor de gewrichtsvernietiging wordt beïnvloed.

In **Hoofdstuk 2** hebben we geprobeerd het verband tussen overmatige IL-1-signalering en de vatbaarheid voor RA-ontwikkeling beter te begrijpen. Botvernietiging tijdens RA wordt grotendeels bemiddeld door zogenaamde osteoclasten, cellen met meerdere
celkernen die ontstaan uit voorlopercellen uit het beenmerg. Om de effecten van overmatige IL-1-signalering op de samenstelling van drie verschillende populaties van osteoclast-voorlopercellen op verschillende skeletplaatsen (lang bot, schedel, wervel en onderkaak), de effecten op de ontwikkeling van osteoclasten hieruit in het lab en uiteindelijk op botresorptie te kunnen onderzoeken, hebben we gebruik gemaakt van genetisch gemodificeerde muizen zonder een 'rem' die de inflammatoire effecten van IL-1 blokkeert. We hebben aangetoond dat overmatige IL-1-signalering sommige populaties van osteoclastvoorlopers aanwezig in het beenmerg van lange botten en onderkaak groter maakte en daarom mogelijk op die plaatsen een toename van osteoclastvorming kan veroorzaken, wat samenviel met een grotere activering van aangeboren immuuniteit. Het blijkt dat in vitro botresorptie onafhankelijk van de skeletplaats van oorsprong van de osteoclasten wordt verhoogd. Onze resultaten suggereren dat de uitbreiding van osteoclastvoorlopers in het lange bot en de onderkaak een mechanisme kan zijn dat ten grondslag ligt aan botvernietiging bij AR en parodontitis.

Een andere cruciale factor voor de activering van het aangeboren immuunsysteem is de interactie tussen Fcy-receptoren die aanwezig zijn op aangeboren immuuncellen en antistoffen van het IgG type en complexen van deze antistoffen, de zogenaamde immuno-complexen (IC’s), die zich ophopen in de gewrichten van patiënten met RA. De expressie van Fcy-receptoren, die vaak veranderd is in het gewrichtsweefsel van patiënten met RA, speelt een belangrijke rol bij de ontwikkeling van de ziekte. De rol van individuele receptoren in de gewrichtspathologie is in het verleden onderzocht in verschillende experimentele modellen van artritis. Het effect van de volledige afwezigheid van alle Fcy-receptoren, en met name de rol van de Fcy IV-receptor in botvernietiging en de verantwoordelijke mechanismen, moest echter nog worden opgehelderd. Daarom hebben we in Hoofdstuk 3 muizen waarin Fcy-receptoren I, II, III, IV niet aanwezig zijn vergeleken met wildtype (WT) -controles die deze Fcγ-receptoren allemaal wel hebben wat betreft het ontstaan van botvernietiging tijdens RA. De volledige afwezigheid van Fcy-receptoren leidde tot een vermindering van boterosie en osteoclast activiteit in vergelijking met WT-muizen.. Door vervolgens de ontwikkeling van artritis te vergelijken tussen de Fcy I-, II- en III-receptor-deficiënte muizen en hun WT-controles vonden we dat, in tegenstelling tot de muizen die alle vier Fcy-receptoren missen, de muizen die alleen de Fcy IV-receptor tot expressie brachten een toename hadden van boterosie, gewrichtsontsteking en het aantal neutrofielen in deze gewrichtsontsteking. Dit suggereert een cruciale rol van de Fcy IV-receptor bij rekrutering van neutrofielen tijdens experimentele artritis. Bovendien was boterosie significant gerelateerd aan het aantal neutrofielen en positieve cellen voor expressie van S100A8 in het gewricht. Deze resultaten suggereren dat activering van Fcy-receptoren
een cruciale rol speelt in de ontwikkeling van boterosie tijdens experimentele artritis door ontsteking te induceren, en in het bijzonder bemiddelt de Fcy IV-receptor boterosie door de instroom van neutrofielen te stimuleren. Deze neutrofielen produceren S100A8/A9 in het artritische gewricht. Verder suggereert dit dat het beperken van de expressie van Fcy-receptoren een veelbelovende therapeutische benadering zou kunnen zijn om botvernietiging veroorzaakt door IC-vorming bij RA-patiënten te verminderen.

Patiënten met RA lijden vaak aan dyslipidemie, gekenmerkt door hoge concentraties van LDL-cholesterol en lagere hoeveelheden HDL-cholesterol. Hoewel algemeen wordt aangenomen dat LDL-hypercholesterolemie het risico op hart- en vaatziekten bij RA-patiënten verhoogt, moet nog worden vastgesteld of een hoge concentratie van LDL-cholesterol op zichzelf artritis verergerd. Paradoxaal genoeg vertonen patiënten met RA met lage spiegels van LDL-cholesterol meer progressie van de ziekte en een groter risico op het ontwikkelen van hart- en vaatziekten, wat aanleiding geeft tot de zogenaamde "lipidenparadox". In een ontstoken gewricht worden hoge spiegels van LDL-cholesterol snel geoxideerd tot oxLDL, dat voornamelijk wordt opgenomen door macrofagen. De stapeling van oxLDL veroorzaakt de productie van vele ontstekingsstimulerende en ontstekingsremmende factoren, waardoor de activiteit van aangeboren immuun cellen wordt beïnvloed. Omdat dit een impact kan hebben op de ontwikkeling van artritis, hebben we in Hoofdstuk 4 en Hoofdstuk 5 het effect bestudeerd van hoge spiegels van LDL-cholesterol en oxLDL op gewrichtsontsteking en gewrichtsvernietiging tijdens experimentele artritis met muizen zonder apolipoproteïne E (APOE), die spontaan hypercholesterolemie ontwikkelen. In Hoofdstuk 4 zien we een vermindering van ontsteking en kraakbeenschade bij muizen met hoge niveaus van LDL-cholesterol na inductie van een experimenteel model van artritis. Daarentegen hadden hoge niveaus van LDL-cholesterol die aanwezig waren in muizen zonder artritis geen effect op ontsteking of kraakbeenvernietiging. Vervolgens hebben we de effecten bestudeerd van het hoge niveau van LDL-cholesterol/oxLDL op de hoeveelheid Fcy-receptoren op macrofagen, omdat tijdens dit experimentele model van artritis veroorzaakt door IC de Fcy-receptoren op macrofagen een cruciale rol spelen. Na te hebben ontdekt dat oxLDL, maar niet LDL-cholesterol, de expressie van activerende Fcy-receptoren verlaagde, suggereert dit dat hoge niveaus van LDL-cholesterol en hun verhoogde oxidatie in het gewricht van de artritis, ontsteking en daaropvolgende vernietiging onderdrukken van kraakbeen door de macrofaag-expressie van Fcy-receptoren te reguleren.

In Hoofdstuk 5 hebben we het effect van hoge niveaus van LDL-cholesterol/oxLDL op de vorming van osteoclasten en bot erosie nader onderzocht. De inductie van experimentele artritis resulteerde in een sterke vermindering van botvernietiging bij
hypercholesterolemische muizen in vergelijking met WT-controles en ging gepaard met een vermindering van het aantal bot-osteoclasten. Omdat de afwezigheid van Apoe het aantal osteoclastvoorlopers in het beenmerg noch de vorming van osteoclasten verlaagde, suggereert dit dat de blootstelling van pre-osteoclasten en osteoclasten aan oxLDL, maar niet aan LDL-cholesterol een sterk remmende werking heeft op de vorming van osteoclasten en hun resorptiecapaciteit. Het is interessant om op te merken dat dit samenviel met een sterke vermindering van genexpressie van factoren die osteoclastogenese stimuleren. Samengevat suggereren deze resultaten dat in het ontstoken gewricht hoge niveaus van LDL-cholesterol botvernietiging verminderen tijdens experimentele artritis, waarschijnlijk vanwege de vorming van oxLDL dat de vorming en activiteit van osteoclasten remt door modulatie van factoren die een belangrijke rol spelen bij de osteoclastvorming.

Naast een belangrijke rol in de regulatie van lipide transport en -accumulatie, is het APOE eiwit ook betrokken bij tal van immunologische processen. Verder hebben eerdere studies aangetoond dat verschillende vormen van het menselijke ApoE-gen (APOE-ε2, APOE-ε3 en APOE-ε4) geassocieerd zijn met de ernst van verschillende ontstekingsziekten zoals atherosclerose en de ziekte van Alzheimer waarbij ontsteking en de activering van het aangeboren immuunsysteem een belangrijke rol speelt. Associatiestudies tussen de aanwezigheid van deze kleine veranderingen in het ApoE-gen en het risico op het ontwikkelen van RA hebben tegenstrijdige resultaten opgeleverd. Een eerdere studie toonde aan dat de APOE-ε4-variant van het ApoE-gen minder frequent was bij patiënten met RA dan bij gezonde personen in vergelijking met de andere twee varianten, hetgeen een beschermende rol van APOE4 bij RA suggereert. Andere studies hebben aangetoond dat APOE4 wordt geassocieerd met ernstigere dyslipidemie en als zodanig de aangeboren immuunrespons en dus de ontwikkeling van RA kan beïnvloeden. Omdat we hebben vastgesteld dat hoge niveaus van LDL-C gewrichtsschade verminderen in experimentele AR, kan het zijn dat proefpersonen met het APOE-ε4-genotype worden beschermd tegen de ontwikkeling van RA, waarschijnlijk vanwege het onderdrukkende effect van oxLDL in de gewrichtsaandoening. Hoewel de betrokkenheid van APOE-varianten redelijk is bestudeerd met betrekking tot het risico op het ontwikkelen van RA, is er weinig bekend over hun invloed op andere reumatische aandoeningen waarbij aangeboren immuuniteit een even belangrijke rol speelt, zoals artrose. Daarom hebben we in Hoofdstuk 6 het effect van twee APOE-varianten (APOE-ε3 en APOE-ε4) op de ontwikkeling van artrose bestudeerd. In deze studie hebben we een experimenteel model van artrose geïnduceerd met behulp van muizen waarbij het Apoe-gen van de muis werd vervangen door de menselijke variant. We hebben vastgesteld dat APOE-ε4-muizen meer ontsteking, grotere kraakbeen- en overmatige botvorming ontwikkelden dan APOE-ε3-muizen, wat suggereert dat APOE-genotypen een risicofactor voor de ontwikkeling van artrose kunnen zijn.
Botvernietiging is een van de belangrijkste kenmerken van RA als gevolg van de toename van het aantal en de activiteit van osteoclasten, zoals benadrukt in de vorige hoofdstukken. Momenteel worden voor onderzoeken naar osteoclasten vaak cellen afkomstig uit het beenmerg van muizen gebruikt, waarvoor veel dieren nodig zijn en dan alsnog is er maar een beperkt aantal beschikbare cellen. Daarom hebben we in Hoofdstuk 7 het gebruik van de ER-Hoxb8-cellijn van monocyt/macrofaag-voorlopercellen gevalideerd voor differentiatie in functionele osteoclasten. In deze studie hebben we beenmergcellen van WT-muizen gebruikt als gouden standaard om mee te vergelijken. Het doel was om een klonale ER-Hoxb8-cellijn te produceren die zowel qua uiterlijk als functie sterk lijkt op osteoclasten afkomstig van muizenbeenmerg. Bovendien is CRISPR/Cas9-technologie toegepast om aan te tonen dat dit model kan worden gebruikt om specifieke genen te inactiveren. Om het principe van deze techniek te tonen, hebben we ER-Hoxb8-cellen gegenereerd waarin de expressie van genen die belangrijk zijn tijdens de ontwikkeling van osteoclasten werd geïnactiveerd, wat er inderdaad toe leidde dat deze cellen niet konden differentiëren naar osteoclasten. Concluderend vertegenwoordigt deze methode een efficiënt en zeer specifiek systeem voor het genereren van een potentieel onbeperkt aantal genetisch gemodificeerde osteoclastvoorlopers voor de in vitro studie van het gedrag van osteoclasten in zowel fysiologische als inflammatoire aandoeningen.

Samengevat geven de resultaten in dit proefschrift meer kennis over hoe verschillende factoren het aangeboren immuunsysteem activeren, en aldus bijdragen aan de vernietiging van weefsels tijdens RA. Verder benadrukt dit proefschrift dat met name de ontwikkeling van immunotherapieën gericht op het blokkeren van activerende Fcy-receptoren en een beter begrip van de klinische effecten van verhoogde LDL-cholesterol-waarden kunnen leiden tot effectievere therapieën voor de behandeling van RA-patiënten die niet reageren op therapieën die nu op de markt zijn.
Sintesi
L’artrite reumatoide (AR) è una malattia infiammatoria cronica e autoimmune delle articolazioni che colpisce lo 0,5-1% della popolazione mondiale. Il protrarsi dell’infiammazione sia a livello sistemico che locale nelle articolazioni provoca l’attivazione cronica del sistema immunitario, portando a un progressivo danno della cartilagine e delle ossa e, infine, alla disfunzione articolare. Sebbene sia noto che molti fattori contribuiscano allo sviluppo della malattia la causa esatta non è del tutto nota. Granulociti, monociti, macrofagi, osteoclasti e i loro precursori, sono cellule dell’immunità innata che svolgono un ruolo centrale nel modulare la distruzione articolare causata dall’infiammazione. Pertanto, ulteriori ricerche aiuterebbero a identificare nuovi meccanismi e fattori coinvolti nello sviluppo della malattia. Ciò potrebbe portare alla scoperta di nuove terapie atte ad interferire con lo sviluppo della risposta immunitaria innata prevenendo così l’istaurarsi di un circolo vizioso e consentirebbe di trattare la malattia in fase precoce e di migliorare significativamente le condizioni di vita dei pazienti.

Scopo della tesi
In questa tesi abbiamo studiato l’impatto dell’immunità innata sulla distruzione articolare nell’artrite reumatoide (AR). La malattia parodontale e la sindrome metabolica sono due importanti esempi di comorbidità dell’AR accomunate dell’attivazione della risposta immunitaria innata. Poiché l’attivazione persistente del sistema immunitario innato è il filo conduttore di queste comorbidità infiammatorie, una comprensione più profonda dei diversi meccanismi alla base di questa iperattivazione cronica aiuterebbe a sviluppare trattamenti più efficaci. Nel complesso, in questa tesi abbiamo studiato gli effetti di vari fattori sull’attivazione delle cellule immunitarie innate come i macrofagi, gli osteoclasti e i loro precursori, e la loro influenza sullo sviluppo dell’AR. In particolare, abbiamo approfondito gli effetti pro-infiammatori dell’interleuchina (IL) -1 e dell’allarmina S100A8/A9, nonché il ruolo dei recettori Fc gamma (Fcγ), tutti fattori che inducono fortemente l’infiammazione, e altresì’ fattori sistemici come il colesterolo lipoproteico a bassa densità (LDL-C) e il LDL-C ossidato (oxLDL) che promuovono l’attivazione dell’immunità innata, influenzando così la distruzione articolare.

Nel **Capitolo 2** abbiamo cercato di comprendere meglio il legame tra l’eccessiva segnalazione dell’IL-1 e la suscettibilità allo sviluppo dell’AR. La distruzione ossea è ampiamente mediata dagli osteoclasti, cellule multinucleate deputate alla degradazione dell’osso che si differenziano da popolazioni di precursori degli osteoclasti residenti nel midollo osseo. Per esplorare gli effetti di un’eccessiva segnalazione dell’ IL-1 sulla composizione di tre diverse popolazioni di precursori degli osteoclasti in diversi siti
scheletrici (osso lungo, calvaria, vertebra e mandibola), nonché i suoi effetti sull’osteoclastogenesi in vitro e sul riassorbimento osseo, abbiamo usato topi geneticamente modificati privi di un ‘freno’ che blocca gli effetti infiammatori dell’IL-1. L’eccessiva segnalazione dell’IL-1 ha comportato l’espansione di alcuni sottoinsiemi di precursori di osteoclasti presenti nel midollo osseo di ossa lunghe e mandibola e pertanto può causare un aumento della formazione di osteoclasti in quei siti scheletrici e portare a una maggiore attivazione di vie di segnalazione dell’immunità innata coinvolte nella distruzione ossea. È interessante notare che il riassorbimento osseo in vitro è aumentato indipendentemente dal sito scheletrico di provenienza degli osteoclasti, sottolineando che le differenze nell’attività di riassorbimento osservate in vivo in condizioni infiammatorie possono essere correlate alle differenze nel tipo di osso. Questi risultati suggeriscono che l’espansione dei precursori degli osteoclasti nell’osso lungo e nella mandibola può rappresentare un meccanismo alla base della distruzione ossea nell’AR e nella malattia parodontale.

Un altro fattore cruciale per l’attivazione del sistema immunitario innato è l’interazione tra i recettori Fcγ presenti sulle cellule immunitarie innate e le immunoglobuline G (IgG) contenenti immunocomplessi (IC) che si accumulano nell’articolazione dei pazienti con AR. L’espressione dei recettori Fcγ, spesso alterata nei pazienti con AR, ha un ruolo rilevante nello sviluppo della malattia. Inoltre, il ruolo patogenico dei singoli recettori è stato studiato in vari modelli sperimentali di artrite. Tuttavia, resta da chiarire l’effetto della completa assenza di tutti i recettori Fcγ, e in particolare il ruolo del recettore Fcγ IV nella distruzione ossea e dei meccanismi responsabili. Pertanto, nel Capitolo 3 abbiamo confrontato topi deficienti per i recettori Fcγ I, II, III, IV e i relativi controlli wild-type (WT) per studiare l’effetto dell’assenza di tutti i recettori Fcγ sulla distruzione ossea in AR. La completa assenza dei recettori Fcγ ha portato ad una riduzione dell’erosione ossea e dell’attività degli osteoclasti rispetto ai topi WT. Ciò è stato il risultato di una diminuzione dell’inflammazione articolare, anche se la risposta immunitaria non è stata compromessa. Dal confronto dello sviluppo dell’artrite tra i topi deficienti per i recettori Fcγ I, II e III e i loro controlli WT abbiamo scoperto che a differenza dei topi deficienti per tutti e quattro i recettori Fcγ, i topi che esprimevano solo il recettore Fcγ IV ha un aumento dell’erosione ossea, dell’inflammazione e del numero di neutrofili. Ciò suggerisce un ruolo cruciale del recettore Fcγ IV nel reclutamento di neutrofili durante l’artrite sperimentale. Inoltre, l’erosione ossea era significativamente correlata al numero di neutrofili e di cellule positive per l’espressione di S100A8 presenti nell’articolazione. Complessivamente, questi risultati suggeriscono che l’attivazione dei recettori Fcγ svolge un ruolo cruciale nello sviluppo dell’erosione ossea durante l’artrite sperimentale inducendo infiammazione, e in particolare, il recettore Fcγ IV media l’erosione ossea stimolando l’afflusso di neutrofili che producono S100A8/A9 nell’articolazione artritica. Inoltre, ciò suggerisce che limitare l’espressione dei recettori
Fcγ potrebbe essere un approccio terapeutico promettente per attenuare la distruzione ossea causata dalla formazione di IC nei pazienti con AR.

I pazienti con AR spesso soffrono di dislipidemia, caratterizzata da alti livelli di LDL-C e colesterolo lipoproteico ad alta densità (HDL-C). Mentre è comunemente accettato che l'ipercolesterolemia LDL aumenta il rischio di malattie cardiovascolari nei pazienti con AR, è ancora da accertare se un alto livello di LDL-C di per sé aggravhi l'artrite. Paradossalmente i pazienti con AR aventi bassi livelli di LDL-C mostrano una maggiore progressione della malattia e un rischio maggiore di sviluppare malattie cardiovascolari, dando luogo al cosiddetto "paradosso lipidico". In un'articolazione infiammata, alti livelli di LDL-C vengono rapidamente ossidati in oxLDL, che viene assorbito principalmente dai macrofagi. L'accumulo di oxLDL innesca la produzione di molti fattori pro-infiammatori e anti-infiammatori, modulando così l'attività delle cellule immunitarie innate. Poiché ciò potrebbe avere un impatto sullo sviluppo dell'artrite, nel Capitolo 4 e nel Capitolo 5, abbiamo studiato l'effetto di alti livelli di LDL-C/oxLDL sull'infiammazione e la distruzione articolare durante l'artrite sperimentale utilizzando topi privi dell'apolipoproteina E (APOE), che sviluppano spontaneamente ipercolesterolemia. Nel Capitolo 4, abbiamo osservato una riduzione dell'infiammazione e dei danni alla cartilagine nei topi con alti livelli di LDL-C dopo induzione di un modello sperimentale di artrite. Al contrario, alti livelli di LDL-C presenti nei topi senza artrite non hanno avuto alcun effetto né sull'infiammazione né sulla distruzione della cartilagine. Questi risultati suggeriscono che la formazione di oxLDL nell'articolazione infiammata, ma non di livelli elevati di LDL-C, influenzano l'insorgenza e la progressione dell'infiammazione e distruzione della cartilagine durante l'artrite sperimentale. Successivamente, abbiamo studiato in vitro gli effetti dell'elevato livello di LDL-C/oxLDL sull'espressione macrofagica dei recettori Fcγ poiché durante questo modello sperimentale di artrite causato da IC, i recettori Fcγ presenti sui macrofagi svolgono un ruolo cruciale. Avendo scoperto che l’oxLDL, ma non LDL-C, ha abbassato l’espressione dei recettori Fcγ attivanti, ciò suggerisce che alti livelli di LDL-C e la loro maggiore ossidazione nell’articolazione artritica, sopprimono l’infiammazione e la conseguente distruzione della cartilagine regolando l’espressione macrofagica dei recettori Fcγ.

Nel Capitolo 5, abbiamo studiato più dettagliatamente l'effetto di alti livelli di LDL-C/oxLDL sull’osteoclastogenesi e sulla distruzione ossea. L'induzione dell'artrite sperimentale ha comportato una forte riduzione della distruzione ossea nei topi ipercolesterolemici rispetto ai controlli WT ed è stata accompagnata da una riduzione del numero di osteoclasti sull’osso. Se l'assenza di Apoe non ha abbassato né il numero di precursori degli osteoclasti nel midollo osseo né la formazione di osteoclasti in vitro, al contrario l’esposizione di pre-osteoclasti e osteoclasti a oxLDL, ma non di LDL-C, ha
fortemente inibito la formazione di osteoclasti e la loro capacità di riassorbimento. È interessante notare che ciò è coinciso con una forte riduzione dell’espressione genica di fattori che stimolano l’osteoclastogenesi. Nell’insieme, questi risultati suggeriscono che nell’articolazione infiammata, alti livelli di LDL-C riducono la distruzione ossea durante l’artrite sperimentale, probabilmente a causa della formazione di oxLDL che inibisce la formazione e l’attività degli osteoclasti attraverso la modulazione i fattori che svolgono un ruolo importante nell’osteoclastogenesi.

Oltre ad avere un ruolo importante nella regolazione del trasporto e dell’accumulo di lipidi, APOE è anche coinvolta in numerosi processi immunologici. Infatti, studi precedenti hanno dimostrato che forme leggermente diverse del gene umano di ApoE (APOE-ε2, APOE-ε3 e APOE-ε4) sono associate alla gravità di diverse malattie infiammatorie come l’aterosclerosi e il morbo di Alzheimer dove l’infiammazione e l’attivazione del sistema immunitario innato svolgono un ruolo importante. Studi di associazione tra la presenza di queste piccole variazioni del gene ApoE e il rischio di sviluppare AR hanno generato risultati contrastanti. Uno studio precedente aveva scoperto che la variante APOE-ε4 era meno frequente nei pazienti con AR rispetto ai soggetti sani paragonato alle altre due varianti. Questi risultati suggeriscono un ruolo protettivo di APOE4 nell’AR. Altri studi hanno dimostrato che APOE4 è associato a dislipidemia più grave e come tale può influenzare la risposta immunitaria innata e quindi lo sviluppo di AR. Avendo osservato che elevati livelli di LDL-C riducono il danno articolare nella AR sperimentale è probabile che i soggetti con il genotipo APOE-ε4 siano protetti contro lo sviluppo della AR a causa dell’effetto soppressivo di oxLDL presente nell’articolazione artritica.

Mentre il coinvolgimento delle varianti di APOE è stato moderatamente studiato rispetto al rischio di sviluppare AR, si sa molto poco della loro influenza su altre malattie reumatiche come l’osteoartrite (OA) dove l’immunità innata svolge un ruolo altrettanto importante. Pertanto, nel Capitolo 6 abbiamo studiato l’effetto di due varianti di APOE (APOE-ε3 e APOE-ε4) sullo sviluppo dell’OA. In questo studio, abbiamo indotto un modello sperimentale di OA su topi il cui il gene Apoe è stato sostituito dalla variante umana. Abbiamo osservato che i topi APOE-ε4 sviluppano più infiammazione, un maggiore danno alla cartilagine, e un’eccessiva formazione di osso rispetto ai topi APOE-ε3. Questi risultati suggeriscono che i genotipi APOE possono rappresentare un fattore di rischio per lo sviluppo di OA.

La distruzione ossea è una delle principali caratteristiche dell’AR causata dall’aumento del numero e dell’attività degli osteoclasti come sottolineato nei capitoli precedenti. Attualmente, gli studi in vitro sugli osteoclasti di topo vengono condotti principalmente su cellule derivate da midollo osseo impattando negativamente sul numero di animali utilizzati e sul numero limitato di cellule disponibili, nonché sull’eterogeneità della fonte. Pertanto, nel Capitolo 7 abbiamo utilizzato la linea cellulare ER-Hoxb8 di cellule progenitrici derivate da monociti/macrofagi per la differenziazione in osteoclasti. In
questo studio abbiamo utilizzato cellule del midollo osseo di topi WT per produrre una linea cellulare clonale ER-Hoxb8 che ricorda fortemente sia la morfologia sia la funzione degli osteoclasti derivati da midollo osseo. Inoltre, la tecnologia CRISPR/Cas9 è stata applicata per dimostrare che questo modello cellulare può essere utilizzato per inattivare specifici geni. Più specificamente, abbiamo generato cellule ER-Hoxb8 in cui è stata inattivata l’espressione di geni che sono importanti durante l’osteoclastogenesi e pertanto queste cellule non riescono a differenziare in osteoclasti. Questo metodo rappresenta un sistema efficiente e altamente specifico per generare un numero, potenzialmente illimitato, di precursori degli osteoclasti geneticamente modificati. Lo studio in vitro di tali cellule permette di analizzare il comportamento degli osteoclasti in condizioni sia fisiologiche che infiammatorie.

In conclusione, i risultati presentati in questa tesi forniscono un’ulteriore conoscenza su come vari fattori scatenanti attivino il sistema immunitario innato, contribuendo così alla distruzione articolare durante l’AR. Inoltre, questa tesi evidenzia in particolare che lo sviluppo di immunoterapie mirate a bloccare i recettori Fcγ attivanti e una migliore comprensione degli effetti clinici di elevati livelli di LDL-C porteranno a terapie più efficaci per il trattamento di pazienti con AR che non rispondono alle terapie attuali.
List of publications

**High LDL levels lessen bone destruction in antigen-induced arthritis by inhibiting osteoclast formation and function.**


**High LDL-C levels attenuate onset of inflammation and cartilage destruction in antigen-induced arthritis.**


**Fcγ receptor-mediated influx of S100A8/A9-producing neutrophils as inducer of bone erosion during antigen-induced arthritis.**


**S100A8/A9 increases the mobilization of pro-inflammatory Ly6C<sup>high</sup> monocytes to the synovium during experimental osteoarthritis.**


**Genetic modification of ER-Hoxb8 osteoclast precursors using CRISPR/Cas9 as a novel way to allow studies on osteoclast biology.**


**S100A8/A9, a potent serum and molecular imaging biomarker for synovial inflammation and joint destruction in seronegative experimental arthritis.**

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I am very grateful to Teun de Vries for the supervision received during the course of our fruitful collaboration! Thanks for making time for me everytime I came to ACTA for our project. You were always available and enthousiastic to discuss science and your deep knowledge about osteoclast biology has been an invaluable tool we all benefit from within Euroclast!
Ineke Jansen thanks a lot for helping with this project and for being always responsive with answering my questions!

Thanks to the rest of the Euroclast buddies! Sara, Viktē, Laia, Anh, Emma, David, Henrik, Sandra, Arjen we had great fun during the meetings and conferences and I will always cherish these moments! What was meant to be an opportunity of professional network soon revealed to be so much more and I hope we’ll be able to plan a reunion somewhere around the world soon!

Thanks to Kim Henriksen, who was my mentor within Euroclast. Our discussions definitely helped me with development plan of my project. Thanks also to Christian Thudium for the mentoring during the Euroclast meetings. I very much appreciated the advice you gave me!

I am very grateful to all people that participated to the Euroclast consortium, who made this experience unforgettable not only for the outstanding training received, but also for the all the beautiful moments we shared. A special thanks to Miep Helfrich and Vincent Everts for believing so much in this consortium. Since the beginning you have been great supporters of us all! Euroclast would have not been the same without you! A big thanks also to the rest of the Euroclast crew, Göran Anderson, Markus Damme, Paul Heftig, Bernard Hoflack, Petra Henning, Ulf Lerner, Fraser Coxon and Tiina Laitala-Leinonen.

Thanks to Bas Overbeek for organizing the Euroclast meetings!

A biiig thanks to my Paranymphs!

Annet(je) aka Mother goose What would have I done without you? You were the first person of the group I met and I remember crystal-clear you were so radiant and kind to me! I told myself ‘if the other people in this lab have only half of her personality, that is the place I wanna be!’ Since the beginning I felt so welcomed and understood! Lieve Annet, you were the person I would always go to when I needed to be cheered up (or throw names at something :D), you were always there to listen and comfort me, even when you had your mind wrapped up in your own things. I love your simplicity and the way you cope with things in life, and I will definitely take that with me… I don’t want to become too cheesy now so I will stop! Thank you Netje(s)!

Irene Ce l’abbiamo fatta!! Non basterrebbe una pagina intera per riassumere tutti i momenti passati insieme! Sin dal primo giorno ci siamo supportate, a volte sopportate 😊, come colleghie ma soprattutto come amiche (posso dire con certezza che nessun collega sarebbe rimasto in lab fino alle 3.30 del mattino durante il mio primo studio in vivo!!!). Questo legame è stato percepito un po’ da tutto il lab e sicuramente ci è valso l’appellativo di ‘kleine Italianese’. La tua gentilezza e tenacia mi hanno insegnato molto. Grazie per essere stata un punto di riferimento in questi anni, sono sicura che sapremo tener vivo il rapporto che si è creato anche se non siamo più le ‘guardiane del lab’ e nonostante la distanza. Semplicemente GRAZIE!!
Thanks to Group 4! Niels, Stephanie (Mrs. Always right!), Nik(ke), Edwin, Wouter, Yvonne Bartels, Yvonne van Gemert, Rik we have always been very supportive to each other and this was just great! We shared joys and disappointments (a PhD always comes with both!) and I will cherish that forevermore! DANKJEWEL!

Thanks to all Colleagues who have been part of this adventure! Laurie (thanks for the many chats we had till late in the lab 😊), Ellen (thanks for your contagious cheerfulness and always positive attitude!), Claire (I’ll miss your laughter a lot!), Rebecca, Debbie, Nathalie, Daphne, Renske, Margot, Joyce, Bartijn, Natalia, Shahla, Rogier, Esmeralda, Arjan, Mathijs, Guus, Henk. Some of you left already, some will be finishing up their PhD soon, and others will stay keeping high the reputation of Lab Reuma! Thank you for all the scientific discussion during the lab meetings and of course for the fun (and biertjes) we had during the movie nights, Lab-lympics, Lab days out, and PhD retreats! I will be missing such a cosy environment and all the good vibes! I wish you all the best!

A big thanks to the Technicians! You are surely the backbone of the lab, always keeping things up and running and helping us staying on track with the work load! Birgitte, thanks for all the help with the histology and -of course, for introducing me to the magic of bitter ballen 😊! Monique, thanks for your amazing touch with cutting and help with the Luminex! Elly, thanks for helping whenever I needed it and for the nice chats under the hood!, Onno, factotum and more recently PhD-fellow in the lab, thanks for making the daily lab routine cheerful with your whistles, and of course for saving my samples from the centrifuge several times 😃! Miranda, still part of the ‘Reuma family’ even if you’re now working elsewhere. …and of course thanks to Marianne for being always so thoughtful, helpful and sound with respect to everyone’s needs.

Dear International friends, this journey would have not been the same without you!! Most of us were far away from home and we sort of took care of each other during the past years. PhD years can be stressful (..just kidding, they definitely are) and having you has been the sweetest treat I could have hoped for. Thanks for all the moments we shared together, travelling, laughing, whining, BBQs and evenings out drinking or dancing things out. THANKS Dana (now’s your turn, dear friend!), Riccardo (Richard), Sarah, Antonio (Toninho), Antoine (Habibi), Bart, Stefania, Davide, André, Cindy, Angela, Antonella, Alessia, Eligio, Omar, Julio. Thanks to the big Biomaterial family, Sonia, Irene, Mani, Nathan, Arminé, Winston, Doris: Y’all made my years and you will be very much missed!!!

Thanks to all members of the playback theatre group Blending voices! This group meant so much to me and many of you know that! Daria, a biig thanks to you and the credit for motivating me singing out loud! I hope we’ll be able to catch up
soon! Ana and Cristina, I will miss a lot playing with you, but even more all the ‘wine and talk’ nights we had! Thanks to Johannet, Rocio, Dries, Ruud, Anchel (hopefully more Boomshakalaka nights to come!!), Elena, Alexandra, Bart, Marc. We shared joys and tears and a lot of deep and meaningful moments that helped me bringing out certain aspect of my personality (I even didn’t know I had). This has been a space-stastic journey in the journey!

Grazie agli Acoustic Blend! We met with a Goodbye k(a)ss(a) ma è stato amore a prima vista! :D Daniela, Alessandro, Eljos, Roberta, Giulia, Mariachiara (fedele sostenitrice e fan 😊!) Grazie per le serate passate insieme a fare baccano! Grazie per aver rallegrato le serate di ‘muddura calatina’ e per la complicità che si è creata in così poco tempo! Vi aspetto a DC!!

Grazie agli Amici di Parma perché anche se dispersi in vari angoli d’Europa non ci siamo mai persi! Erika, Riccio, Pam, Fabiola, Giulia, Fabio, Davide, Gervo, Stacchio grazie per il tempo passato insieme e per tutte le reunions organizzate...Next stop: USA!!! Gli amici che vuoi sono i soli che avrai, ed io ho scelto bene! 😊

Marialuisa, Lov grazie per esserci sempre stata e per essere quella persona speciale che anche se si è lontane quando ci si rivede sembra il tempo sembra essersi fermato! Grazie perché la nostra amicizia ha resistito al tempo e alla distanza!

David, Merci mon frère parce-que même si on à été longtemps éloignés on a sù se rattrapper! Merci pour la confiance, le soutien et l’encouragement que tu m’as donnés dans les difficultés pendant ces années de doctorat.

Simone, Morosito, a te va un ringraziamento speciale per essere stato la mia roccia in questi anni! Come avrei fatto senza di te?! Grazie per avermi sostenuta, capita, consigliata, spronata, rassicurata...e sopportata! Non vedo l’ora di iniziare una nuova tappa del nostro viaggio insieme!!

Un enorme grazie alla mia Famiglia!
Grazie zii Marisa, Aurora, Michele, ed Emanuele per avermi sempre supportata e perché ognuno di voi mi ha insegnato il valore della determinazione e della perseveranza. Chi la dura la vince! Grazie Daniela (Daddi) e Andrea (Uccio), sorella e fratello per scelta, perché seppur lontani dagli occhi siamo sempre rimasti vicini al cuore!

Mamma, questa tesi la dedico a te! Grazie per tutti i sacrifici fatti e che mi hanno permesso di arrivare fin qui! Grazie per avermi insegnato a stare al mondo e per aver sempre sostenuto le mie scelte. Grazie perché non mi hai mai fatto pesare la distanza, anzi mi hai sempre spronata a fare ciò in cui credevo. Grazie per avermi insegnato il valore dell’impegno, e che ogni tanto bisogna osare, altrimenti si rischia di appiattirsi! Grazie Mommy!

[Signature]

189
**PHD PORTFOLIO**

**Name PhD candidate:** Giuliana Ascone  
**Department:** Experimental Rheumatology  
**Graduate School:** Radboud Institute for Molecular Life Sciences  
**PhD period:** 01-04-2014 – 01-07-2018  
**Promotor:** Prof. Dr. PM van der Kraan  
**Co-promotor(s):** Dr. PLEM van Lent  
Dr. MHJ van den Bosch

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<tr>
<th>TRAINING ACTIVITIES</th>
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<tr>
<td><strong>a) Courses &amp; Workshops</strong></td>
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**TOTAL** 37.5

* = Poster presentation; # = Oral presentation; Euroclast ITN = http://www.euroclast.eu/
Curriculum vitae

Giuliana Ascone was born in Catania, Italy, on 12th May 1987 and raised in the near town of Caltagirone. In 2009 she obtained her BSc Degree in Biotechnology at the University of Parma, Italy. She conducted her BSc thesis on the effects of hydrogen sulphide on keratinocytes for the treatment of psoriasis under the supervision of Dr. Giuliana Gobbi. In 2012 she achieved the MSc Degree in Medical Biotechnology at the University of Parma after completion of her MSc thesis, in which she focused on novel strategies for the in vitro formation of cartilaginous tissue under the supervision of Dr. Roberto Sala. In 2013 she joined Peter Friedl’s group for pre-doctoral fellow at the Department of Cell Biology at the Radboud university medical center (Radboudumc), Radboud Institute for Molecular Life Sciences (RIMLS), Nijmegen, the Netherlands. In 2014 Giuliana was selected for a Marie Curie Initial Training Network (ITN) – Euroclast, and started her PhD project at the Department of Experimental Rheumatology at the Radboudumc, RIMLS, Nijmegen, the Netherlands, under the supervision of Dr. Peter van Lent. During her PhD Giuliana focused on understanding better the mechanisms involved in the interaction between the innate immune system and joint pathology with respect to cartilage and bone destruction in rheumatic diseases. Furthermore, in the frame of Marie Curie ITN, Giuliana received part of her training at Future Diagnostics solutions (FDx), Wijchen, the Netherlands, where she deepened her knowledge about developing in vitro diagnostics (IVD) assays under the supervision of Dr. Ernst Lindhout.

In January 2020 Giuliana will start working as a postdoctoral fellow at the National Human Genome Research Institute (NHGRI), National Institutes of Health (NIH), Bethesda, MD, USA.
‘Non temete i momenti difficili, il meglio viene da lì’

Rita Levi-Montalcini