Universidade de Lisboa
Faculdade de Medicina

Differences in osteoclast activity between rheumatoid arthritis and ankylosing spondylitis

Inês Pedro Perpétuo

Orientadores: Professor Doutor João Eurico Fonseca
Doutora Mari-Mia Ainola

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas
Especialidade em Biologia Celular e Molecular

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À minha Mãe.

" When you open your eyes

When you gaze at the sky

When you look to the stars

As they shut down the night

You know... The story ain't over"

- *'The Story Ain't Over'*, Avantasia, Tobias Sammet

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Acknowledgements

A PhD is a journey that changes you, a life within your life that takes you to some very dark and very bright places in a sequence that surprises you. A PhD in Science is the quest for the answer to THAT question, to the riddle that puzzles you and in the end only more questions arise - thank you Science for teaching me to expect the unexpected.

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List of abbreviations

ACPA - anti-citrullinated protein antibodies

ACR - American college of rheumatology

ANTXR2 - anthrax toxin receptor 2

ALP - alkaline phosphatase

AP-1 - activator protein-1

APRIL - a proliferation-inducing ligand

AS - ankylosing spondylitis

ASDAS - ankylosing spondylitis disease activity score

ATF - activating transcription factor

ATP - Adenosine triphosphate

ATP6V0D2 - ATPase H^+ transporting, lysosomal 38kDa V0 subunit d2

BAFF - B cell activating factor

BGLAP - bone δ-carboxyglutamic acid-containing protein

BASDAI - Bath ankylosing spondylitis disease activity index

BASFI - Bath ankylosing spondylitis functional index

BMD - bone mineral density

BMP - Bone morphogenetic protein

BMU - basic multicellular unit

BRC - bone remodelling compartment

CA - carbonic anhydrase

Cbfa1 - core-binding factor subunit alpha-1

CCL - C-C chemokine ligand

CCR - C-C chemokine receptor

CD - cluster of differentiation

cDCs - conventional dendritic cells

CIA - collagen induced arthritis

CLCN7 - chloride/H^+ antiporter channel 7

COX-2 - cyclooxygenase-2

CSF1R - colony-stimulating factor 1 receptor

CT-1 - cardiotrophin

CTLA4 - cytotoxic T-lymphocyte-associated protein 4

CTX-I - C-terminal cross-linked telopeptides of collagen type I

CXCL - C-X-C motif chemokine

DAP12 - DNAX activation protein of 12 kDa

DAS28 - disease activity score 28

DCs - dendritic cells

DC-STAMP - dendritic cell specific transmembrane protein

DKK1 - dickkopf-related protein 1

DMARDs - disease modifying anti-rheumatic drugs

DPD - deoxypyridinoline

Dsh - dishevelled

DXA - dual-energy X-ray absorptiometry

ERK - extracellular-signal-regulated kinases

EULAR - European league against rheumatism

Fab - fragment antigen-binding

Foxp3 - forkhead box P3

FRA - fos-related antigen

G-CSF - granulocyte colony stimulating factor

GM-CSF - granulocyte-macrophage colony stimulating factor

GPI - glucose-6-phosphate isomerase

GSK-3β - glycogen synthase kinase-3β

HCl - hydrochloric acid

HLA - human leukocyte antigen

HSC - hematopoietic stem cell

hTNFtg - human tumor-necrosis-factor transgenic

ICAM - intercellular adhesion molecule

ICTP - cross-linked carboxyterminal telopeptide of type I collagen

IFN-γ - interferon-gamma

IGF-1 - insulin growth factor

iNKT - invariant natural killer T cells

JNK - c-Jun N-terminal kinase

KO - knockout

LPR - low-density lipoprotein receptor-related protein

LPS - Lipopolysaccharide

MAP2K6 - mitogen-activated protein kinase 6

M-CSF - macrophage colony stimulating factor

MCP-1 - monocyte chemotactic protein

MFR - macrophage fusion receptor

MGP – matrix gla protein

MHC - major histocompatibility complex

miR, miRNA - micro ribonulceic acid

MITF - microphthalmia-associated transcription factor

MMPs - matrix metalloproteinases

MSCs - mesenchymal stem cells

MTX - methotrexate

NFATc1 - nuclear factor of activated T cell c1

NF-κB - nuclear factor-κB

NSAIDs - nonsteroidal anti-inflammatory drugs

NTX-I - type I collagen cross-linked N- telopeptide

OA - osteoarthritis

OBs - osteoblasts

OCN - osteocalcin

OC - osteoclast

OPG - osteoprotegerin

OPN - osteopontin

Osx - osterix

P1CP - C--terminal propeptide of type I procollagen

P1NP - N-terminal propeptide of type I procollagen

PDCD4 - programmed cell death protein 4

pDCs - plasmacytoid dendritic cells

PI3K - phosphatidylinositol-4,5-bisphosphate 3-kinase

PTH - parathyroid hormone

PTPN22 - protein tyrosine phosphatase non-receptor 22

PYD - pyridinoline

RA - rheumatoid arthritis

RANK - receptor activator of nuclear factor-κB

RANKL - receptor activator of nuclear factor-κB ligand

RF - rheumatoid factor

RGD - amino acid sequence - arginin-glycin-aspartic acid

ROS - reactive oxygen species

Runx2 - runt-related transcription factor

S1P - sphingosine-1-phosphate

SCF - stem cell factor

SDF-1 - stromal cell-derived factor

sFRPs - secreted Frizzled related proteins

SNP - single nucleotide polymorphisms

SOST - Sclerostin

SpA - spondyloarthritis

sRANKL - soluble receptor activator of nuclear factor-κB ligand

TACE - tumour necrosis factor-alpha converting enzyme

TCF/LEF - T-cell factor/lymphoid enhancer factor

TCRs - T-cell receptors

Teff - effector T cells

Tfe3 - transcription factor E3

TGF- β - transforming growth factor-β

Th17 - T helper 17 cells

TLR - Toll-like receptor

TNF - tumour necrosis factor

TNFi - tumour necrosis factor inhibitors

TRAF - tumour necrosis factor receptor-associated factor

TRAP - tartrate-resistant acid phosphatase

Treg - regulatory T cells

TREM2 - triggering receptor expressed on myeloid Cells 2

VCAM - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor

WIF - Wnt inhibitory factor

Wnt - wingless-related integration site

Sumário

Em muitas doenças inflamatórias, tais como a artrite reumatóide (AR) e a espondilite anquilosante (EA), as células do sistema imunitário alteram a remodelação óssea. A AR apresenta-se tipicamente como uma poliartrite simétrica, mais comum nas mulheres do que nos homens e está associada à presença de auto-anticorpos no soro, tais como anticorpos anti-proteínas citrulinadas (ACPA - *anti-citrullinated protein antibodies*) e factor reumatóide. Os genes do complexo major de histocompatibilidade II (MHC-II - *major histocompatibility complex*) *human leukocyte antigen* (HLA)-DR estão fortemente associados à doença. A inflamação crónica na AR leva à destruição da cartilagem e do osso, sendo que a AR é reconhecida como uma doença erosiva. Por outro lado, a EA é caracterizada por ser uma doença com envolvimento inflamatório axial, com predomínio pelas articulações sacroilíacas e coluna vertebral. Esta doença é mais prevalente no sexo masculino e é fortemente associada ao HLA-B27. A longo da evolução da doença, os doentes apresentam anquilose das articulações da coluna vertebral e sacro-ilíacas.

Enquanto a AR é uma doença caracterizada pela destruição do osso e cartilagem, na EA assistimos a formação óssea aumentada. Neste trabalho, sugerimos que a incapacidade dos osteoclastos (OC) ou dos seus precursores para responder aos estímulos osteoclastogénicos em doentes com EA contribui para a formação óssea excessiva, característica desta doença. O objetivo deste trabalho foi a caracterização dos precursores circulantes de OC (monócitos) em doentes com AR e EA, bem como a sua capacidade de se diferenciar em OC e a sua actividade de reabsorção quando diferenciados *in vitro*. Para além disso, foi também objectivo desta tese compreender o efeito de terapêuticas, tais como o metotrexato (MTX) e inibidores do factor de necrose tumoral (anti-TNF), nos precursores de OC em doentes com AR e EA.

Em primeiro lugar foram investigados os níveis séricos de citocinas em doentes com AR muito inicial, com menos de 6 semanas de duração da doença (*very early rheumatoid arthritis*, VERA), antes e depois do tratamento com corticosteróides e MTX. As citocinas pró-inflamatórias foram quantificados no soro de doentes VERA e doentes com AR estabelecida e em comparação com outras artrites iniciais (*very early arthritis*, VEA) e controlos saudáveis. Líquido sinovial de doentes com AR e osteoartrose (OA) foi também analizado. Os doentes VERA apresentavam níveis séricos aumentados de citocinas que promovem a polarização Th17 (IL-1β e IL-6), bem como IL-8 e citocinas derivadas de

células Th17 (IL-17A e IL-22) que promovem cronicidade da inflamação. Estas citocinas também estão associadas com a promoção da osteoclastogénese. O tratamento inicial com corticosteróides ou MTX levou a uma melhoria clínica, mas sem um impacto sobre o padrão de citocinas. Este aumento de citocinas relacionadas com a polarização Th17 e a osteoclastogénese numa fase tão inicial da doença, uma alteração também encontrados no líquido sinovial das AR estabelecidas, sugere que um ambiente pro-inflamatório favorecedor da polarização Th17 e da actividade dos OC é um evento precoce na patogénese da AR.

De seguida avaliámos o efeito do MTX nos precursores circulantes de OC e na sua diferenciação em OC em doentes com AR. Foram recrutados dadores saudáveis e doentes com AR antes do tratamento e, pelo menos, 6 meses após a introdução de MTX. A expressão do *receptor activator of nf-kB* (RANK) *ligand* (RANKL) em leucócitos e a frequência e fenótipo de subpopulações de monócitos circulantes foram estudadas, bem como foram quantificados os níveis séricos de marcadores de remodelação óssea. Os percusores circulantes foram diferenciados *in vitro* e a sua actividade foi determinada. O RANKL sérico, marcadores de activação clássicos de monócitos (*C-C chemokine receptor 2* - CCR2, CD86, HLA-DR) e o RANK estavam aumentados em doentes com AR com doença activa, em comparação com dadores saudáveis, e após a exposição ao MTX estes parâmetros normalizaram. Não foram encontradas diferenças no número de OC entre os três grupos, mas as células diferenciadas de doentes com AR apresentaram maior atividade de reabsorção do que as de dadores saudáveis. Mais uma vez, após o tratamento com MTX, a actividade dos OC, medida através de ensaio de reabsorção *in vitro*, foi normalizada, sugerindo assim que o MTX desempenha um papel importante na regulação negativa da função dos OC através da diminuição da expressão de RANK em monócitos.

De seguida o efeito dos anti-TNF na osteoclastogénese em doentes com AR foi estudado. Doentes com AR tratados com anti-TNF foram analisados antes e após um período mínimo de seguimento de 6 meses. Foi realizada uma análise de frequência e fenótipo de subpopulações de monócitos, bem como da expressão de RANKL à superfície de leucócitos. Foram quantificados os níveis séricos de marcadores de remodelação óssea e efectuamos ensaios *in vitro* de diferenciação em OC e expressão génica das células em cultura. Após o tratamento com anti-TNF, a expressão de RANKL em linfócitos B encontrava-se diminuída assim como a frequência de precursores de OC em circulação. *In vitro*, o número de OC após tratamento com anti-TNF estava diminuído e estas células apresentavam também menor actividade de reabsorção. Nestas células observámos também

diminuição da expressão de genes importantes para a osteoclastogénese, como *TNF receptor associated protein 6* (TRAF6), *Fos-2 related antigen* (FRA-2) e para a reabsorção, como a catepsina K. Estes resultados sugerem que na AR a terapêutica anti-TNF diminui a reabsorção óssea através da redução directa do número de precursores em circulação e da inibição das vias de sinalização intracelular que actuam através da TRAF6.

Depois de analisar o comportamento dos precursores circulantes de OC em doentes com AR tratados e não tratados prosseguimos para a segunda parte deste trabalho que teve como objetivo caracterizar a remodelação óssea, o ambiente pró-inflamatório e pro-osteoclastogénico, o fenótipo e frequência de monócitos e a sua diferenciação em OC *in vitro* em doentes com EA.

Doentes com EA activa sem qualquer terapêutica em curso e controlos saudáveis foram recrutados. Os níveis de citocinas pró-inflamatórias estavam elevados em doentes com EA em comparação com dadores saudáveis. Apesar de nao haver diferenças no número de precursores circulantes, a expressão de CD51/CD61 (integrina αvβ3 ou receptor da vitronectina, importante na adesão dos OC à matriz óssea) estava diminuída na subpopulação clássica de monócitos. Não foram observadas diferenças no número de OC formados *in vitro* nem na reabsorção óssea, no entanto, genes essenciais à diferenciação em OC (*colony stimulating factor 1 receptor*, CSF1R; RANK e *nuclear factor of activated T cell c1*, NFATc1), apresentavam menor expressão nos doentes com EA, o que consequentemente levou a uma diminuição da expressão de genes de reabsorção, tais como a catepsina K. Estes resultados mostraram que, apesar dos níveis elevados de citocinas pró-inflamatórias presentes nos doentes com EA, os monócitos circulantes têm menor expressão de genes específicos de OC, o que apoia a nossa hipótese de uma resposta inadequada dos precursores OC a estímulos pró-osteoclastogénicos em doentes com EA.

Para compreender os efeitos do tratamento anti-TNF nos precursores circulantes de OC e na sua capacidade de diferenciação em doentes com EA, seguimos uma coorte de doentes antes e depois do início do tratamento com anti-TNF. Os níveis séricos de IL-23 e IL-17A diminuíram após o tratamento com anti-TNF. O número de OC formados a partir de células de doentes com EA antes do tratamento estava reduzido em comparação com os controlos saudáveis. Não foram encontradas diferenças na frequência dos precursores de OC ou no número de OC formados em cultura após o tratamento com anti-TNF. A expressão de CSF1R e NFATc1 nos precursores circulantes em doentes após o tratamento encontrava-se diminuída quando comparada com os dadores saudáveis. No entanto, quando células de doentes sob anti-TNF foram diferenciadas *in vitro* apresentaram maior

actividade de reabsorção do que as células de doentes antes do tratamento e controlos saudáveis. Estes resultados sugerem que em doentes com EA, o tratamento anti-TNF reduz a resposta a estímulos pró-osteoclastogénicos sistémicos, mas quando os precursores de OC de doentes tratados com anti-TNF são colocados em cultura a sua resposta aos estímulos está aumentada.

Em conclusão, o trabalho discutido nesta tese elucida alguns dos mecanismos pelos quais o MTX e os anti-TNF actuam sobre precursores circulantes de OC em doentes com AR e EA.

Embora a AR e a EA sejam ambam doencas crónicas imunomediadas os seus efeitos no metabolismo ósseo são diferentes. Nesta tese mostramos que os precursores de OC de doentes com AR e EA se comportam de modo diferente *in vitro*, mesmo vindo de um ambiente pró-inflamatório semelhante.

Os nossos resultados apoiam a hipótese de que os OC de doentes com EA têm uma falha na sua actividade quando comparados com células de doentes com AR ou controlos saudáveis. Esta diferença pode ser parcialmente explicada por uma incapacidade intrínseca de resposta a estímulos osteoclastogénicos e pela diminuição da expressão de genes-chave de diferenciação e actividade de OC em doentes com EA.

Palavras chave: Artrite reumatóide, espondilite anquilosante, osteoclasto, monócitos, metotrexato, inibidores do TNF

Summary

In many inflammatory diseases, such as rheumatoid arthritis (RA) and ankylosing spondylitis (AS), bone is a target for immune cells unbalancing bone remodeling. RA typically presents as a symmetric polyarthritis, affecting more women than men and is linked with the presence of autoantibodies in the serum such as anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF). Human leukocyte antigen (HLA)-DR genes are strongly associated with the disease. Chronic inflammation in RA leads to cartilage and bone destruction, which is typically recognized as erosive disease on x-rays. In contrast, AS is characterized by axial disease involving the sacroiliac joints and the spine. AS affects more men than women and is strongly associated with HLA-B27 haplotypes. The long-term outcome is characterized by ankylosis of the spine and sacroiliac joints.

While RA is a disease characterized by destruction of bone and cartilage, the predominant finding in AS is bone formation rather than its destruction. In this work, we hypothesize that the inability of osteoclasts (OC) or its precursors to respond to osteoclastogenic stimuli in AS patients contributes to the excessive bone formation characteristic of this disease. Therefore, the aim of this thesis was the characterization of OC circulating precursors (monocytes) in RA and AS, as well as their ability to differentiate in resorbing OC when cultured *in vitro*. Moreover, we also aimed to understand the effect of therapies, such as methotrexate (MTX) and tumor necrosis factor inhibitors (TNFi), in the OC precursors in RA and AS patients.

We first investigated whether cytokines were dysregulated in very early rheumatoid arthritis patients with less than 6 weeks of disease duration (VERA) before and after treatment with corticosteroids and MTX. Pro-inflammatory cytokines were quantified in the serum of VERA and established RA patients and compared with other very early arthritis (VEA) and healthy controls. Patients were also analyzed after therapy. Synovial fluid (SF) from RA and osteoarthritis (OA) patients was also analyzed. VERA patients had increased serum levels of cytokines promoting Th17 polarization (IL-1β and IL-6), as well as IL-8 and Th17-derived cytokines (IL-17A and IL-22) known to promote the chronicity of inflammation. These cytokines are also associated with the promotion of osteoclastogenesis. In established RA this pattern was more evident within the SF. Early treatment with MTX or corticosteroids led to clinical improvement but without an impact

on the cytokine pattern. VERA patients already display increased levels of cytokines related with Th17 polarization and osteoclastogenesis, a deregulation also found in SF of established RA, suggesting that a cytokine-milieu favoring Th17 and OC activity is an early event in RA pathogenesis.

We then aimed to assess the effect of MTX on circulating OC precursors and OC differentiation in RA patients. RA patients were assessed before therapy and at least 6 months after the introduction of MTX therapy and results controlled with healthy donors. We determined receptor activator of NF-κB (RANK) ligand (RANKL) surface expression on circulating leukocytes and frequency and phenotype of monocyte subpopulations. Quantification of serum levels of bone turnover markers and cytokines and in vitro OC differentiation were also performed. We found that serum RANKL, classical activation monocytes markers (C-C chemokine receptor 2 - CCR2, CD86, HLA-DR) and RANK were increased in RA patients with active disease compared to healthy donors, and after MTX exposure these parameters normalized to control levels. Although we found no differences in OC number, cells differentiated from RA patients showed higher resorption activity than from healthy donors. Again, after MTX treatment, osteoclasts resorption activity was normalized. The results of this work suggested that MTX plays an important role in downregulating OC function through the decrease in RANK surface expression in monocytes.

We then proceeded to study the effect of TNFi in osteoclastogenesis in RA patients. RA patients treated with TNFi were analyzed at baseline and after a minimum follow-up period of 6 months. Results were controlled with healthy donors. After TNFi therapy, RANKL surface expression was downregulated in B lymphocytes and the frequency of circulating OC precursors was also decreased. Cells from TNFi treated patients had decreased osteoclast numbers and resorption activity as well as decreased expression of specific genes important for osteoclastogenesis, like tumour necrosis factor receptor-associated factor (TRAF6), fos-related antigen 2 (FRA-2) and for bone resorption like cathepsin K. Therefore, we suggest that in RA TNFi decreases bone resorption through the direct reduction of the number of circulating precursors and the inhibition of intracellular signalling pathways acting through TRAF6.

After exploring OC precursor behavior in untreated and treated RA patients we aimed to characterize bone remodeling and pro-osteoclastogenesis inflammatory environment, monocytes phenotype and in vitro OC differentiation in AS patients.

Patients with active AS without any ongoing therapy and age and gender matched healthy donors were recruited. We observed that pro-inflammatory cytokine levels were higher in a cohort of untreated AS patients when compared to healthy donors, but CD51/CD61 expression (integrin $\alpha_v\beta_3$ or vitronectin receptor, important for osteoclast attachment to the bone matrix) was downregulated in the classical OC precursors. No differences in the *in vitro* osteoclastogenesis or bone resorption was observed when compared to healthy donors, however we found low expression of colony stimulating factor 1 receptor (CSF1R), RANK and nuclear factor of activated T cell c1 (NFATc1) in AS osteoclast precursors that consequently led to a decreased expression of important resorption genes such as cathepsin K. These findings showed us that despite the high levels of pro-inflammatory cytokines present in AS patients, circulating monocytes have low OC specific gene expression supporting our hypothesis of an impaired response of OC precursors to pro-osteoclastogenic stimuli in AS patients.

In an effort to understand the effects of TNFi therapy in circulating OC precursors and their differentiation ability from AS patients, we followed up a cohort of patients before and after therapy. Results were controlled with healthy donors. We found that IL-17A and IL-23 circulating levels decreased after TNFi treatment. OC number was decreased in AS patients before treatment when compared to control. However, no differences in OC precursor frequency or in the number OC in culture were found after treatment. RANK, CSF1R and NFATc1 expression was downregulated in circulating OC precursors after TNFi treatment. However, when cultured in OC differentiated from AS TNFi-treated patients showed higher resorption activity than cells from patients before treatment. These results showed us that in AS patients, TNFi treatment reduces systemic pro-osteoclastogenic stimuli but when OC precursors are exposed to TNFi therapy they have increased *in vitro* activity in response to osteoclastogenic stimuli.

From this work we were able to understand some of the mechanisms by which MTX and TNFi therapies act on circulating OC precursors in RA and AS patients.

Although RA and AS are two chronic immune mediated diseases their effect on bone metabolism is different. The work here discussed shows that RA and AS OC precursors have a different behaviour *in vitro*, even coming from a similar pro-inflammatory milieu.

Our findings support the hypothesis that OC from AS patients have impairment in their activity when compared both to RA patients or healthy controls. This difference can be partially explained by an intrinsic inability to respond to osteoclastogenic stimuli and by downregulation of key OC differentiation and activity genes in AS patients.

Keywords: Rheumatoid arthritis, ankylosing spondylitis, osteoclast, monocytes, methotrexate, tumour necrosis factor inhibitors

Introduction

Bone

Bone is a mineralized connective tissue that is constantly remodelled to support calcium homeostasis and structural needs. Apart from its mechanical role of sustaining the whole body and allowing locomotion, bone has also many other important functions. It plays a protective role shielding vital organs and structures like the bone marrow and spinal cord, and a metabolic role, regulating the homeostasis of calcium and phosphate. Bone is also a hematopoietic organ, since it is where the bone marrow is found, and an endocrine organ, contributing to the global energy balance and serving as a reservoir of growth factors and cytokines [1-3].

Organization

At the microscopic level, bone is composed of two portions, cortical and trabecular. Cortical bone represents 80% of skeletal bone and is dense and compact with low turnover ratio. This type of bone constitutes the outer part of all bones, providing mechanical strength and protection [4]. The trabecular bone only composes 20% of the whole skeleton and is found inside long bones and in vertebrae, surrounded by cortical bone [3, 5]. This type of bone has a very porous structure and a higher turnover rate and elasticity than cortical bone. Therefore, it is light, quickly adaptable to external biological stimulus, flexible and capable of absorbing energy [4]. The cortical structure, on the other hand, has the ability to tolerate the peak loads that the long bones are subjected to [6].

Microstructure

The bone matrix comprises an organic and an inorganic phase. The organic phase is composed mainly by type I collagen, glycoproteins, proteoglycans and bone cells. On the other hand, the inorganic part is formed by carbonated hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$) that are distributed among the collagen fibres [7-9]**.** The composition and organization of the bone matrix gives this tissue unique mechanical properties such as stiffness, ductility, tensile strength and exceptional lightness [2, 9].

The organic component of bone is comprised mostly by type I collagen (90%) and non-collagenous proteins. Type I collagen is synthesized by osteoblasts. It self-assembles in fibrils and fibres that are deposited in parallel or concentric layers [1, 10]. There are several non-collagenous proteins on bone, such as phosphoproteins, proteoglycans, glycosylated proteins and γ-carboxylated proteins. These include osteocalcin (OCN),

matrix gla protein (MGP), osteopontin (OPN), bone sialoprotein (BSP), alkaline phosphatase (ALP) and osteonectin [3]. These proteins are all involved in the ordered deposition of hydroxyapatite by regulating the amount and size of the mineral crystals [1, 3, 11-13].

Phosphoproteins are proteins that undergo post-translational modifications by the attachment of a phosphate group. They bind calcium and thereby act as mineral nucleators [4]. They include BSP and proteoglycans as minor constituents of the bone matrix. Proteoglycans inhibit calcification by masking the collagen fibrils or occupying critical spaces within the fibril and thereby diminishing diffusion, chemical interaction and sequestration of calcium ions or calcium phosphate complexes [13]. BSP is a significant component of the bone extracellular matrix and has been suggested to constitute approximately 8% of all non-collagenous proteins found in bone [14]. It is only present in bone and it functions as another nucleator of hydroxyapatite as well as providing cell attachment and possibly activation of osteoclasts like OPN [15-17].

OCN, also known as bone δ-carboxyglutamic acid-containing protein (BGLAP), is the most abundant noncollagenous protein in bone comprising about 20% of the noncollagen matrix proteins produced by osteoblasts [10, 15]. It contains three γ-carboxylglutamic acid (Gla) residues that bind calcium, and is vitamin K-dependent. It has been postulated that rather than facilitating calcification it could retard it, and also act as a chemoattractant for osteoclasts [18]. ALP is an enzyme produced by osteoblasts and linked to the mineralization process [19]. This protein may be involved in the degradation phosphate esters to provide a local concentration of phosphate or it may remove pyrophosphate to enable mineralization to proceed [4, 10, 20]. Like ALP, OCN is used clinically as a marker of osteoblast activity and serum osteocalcin is measured by radioimmunoassay as a bone turnover marker [18]. MGP, like OCN, is a member of the vitamin K-dependant Gla proteins that acts as a regulator of extracellular matrix calcification. In fact, evidence showed that MGP-deficient mice exhibit spontaneous calcification of cartilage and arteries [15].

In addition to the above matrix proteins are a number of different cell attachment proteins that have the common RGD amino acid sequence (arginin-glycin-aspartic acid), which mediate attachment of these proteins to integrins (integral membrane proteins) on the cell surface [17, 21]. These cell attachment proteins include fibronectin, OPN, osteonectin, and BSP [14]. Fibronectin is a ubiquitous cell attachment protein synthesized locally but also brought in by the vasculature [13, 22]. It is uncertain if the fibronectin has a special

function in bone other than to coordinate the migration, interaction and differentiation of osteoblast precursors *in vitro* and *in vivo* [23-25]. OPN is produced by osteoblasts, binds to calcium and has also a role in osteoclast attachment and bone resorption [14, 26]. OPN expression is regulated by vitamin D, which increases its secretion. It binds to integrin receptors on the osteoclast by its RGD sequence, increasing the osteoclast intracellular calcium concentration [10, 27]. Osteonectin is an acidic glycoprotein involved in cell attachment. It supports bone remodelling and maintenance of bone mass, as shown by the poor bone quality of osteonectin-deficient mice [28]. Although it is synthesized by osteoblasts, osteonectin is also synthesized by skin fibroblasts, tendon cells and odontoblasts [4]. It binds to type I collagen and to hydroxyapatite, promoting crystal growth in vitro [10, 29].

Bone cells

There are three main cell types present in bone: (i) osteoclasts (OCs), giant multinucleated cells derived from macrophage-monocyte lineage that resorb bone by dissolving the mineral phase and by enzymatically degrading extracellular matrix proteins; (ii) osteoblasts (OBs), cells of mesenchymal origin responsible for bone formation that are able to produce the organic bone matrix and aid in its mineralization; (iii) and osteocytes, that are mature OBs that become entrapped in the bone matrix and act as mechanosensors, a crucial function in the regulation of bone remodelling [8, 30].

Osteoclasts

Osteoclasts are bone resorbing cells, formed by cytoplasmic but not nuclear fusion of mononuclear precursors of the monocyte/macrophage lineage of the bone marrow (hematopoietic stem cells), peripheral circulation and tissue macrophage populations in a process summarized in figure 1 [31-33]. OCs formation, differentiation, function and survival are regulated by a network of signalling pathways and dependent on two major factors: macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (RANK) ligand (RANKL) [32, 34].

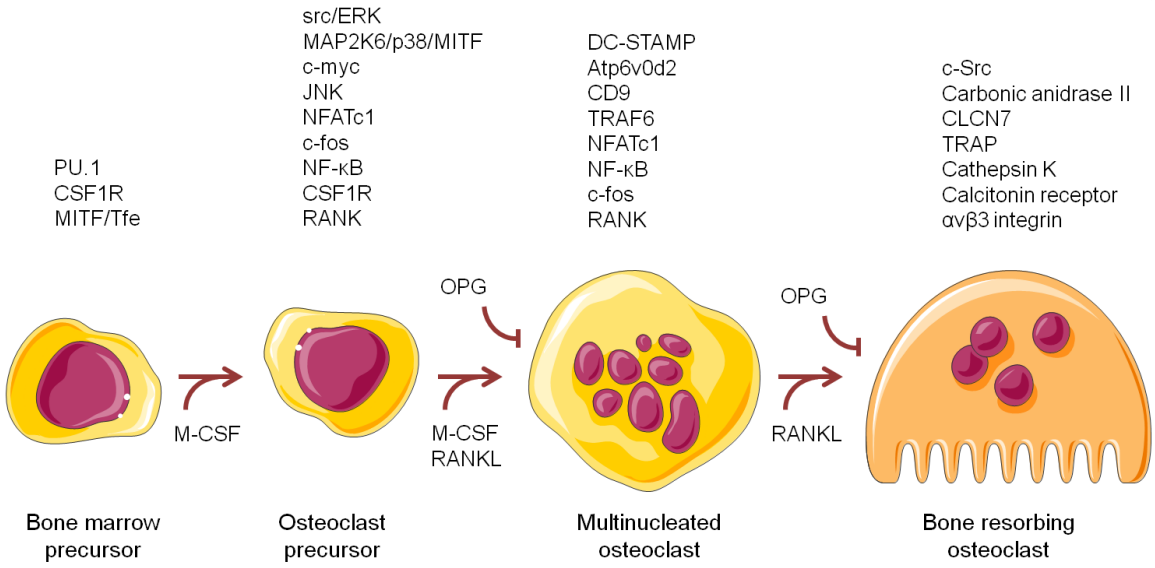

Figure 1 - Osteoclastogenesis. Maturation of osteoclasts occurs in bone either from a bone marrow precursor or from peripheral blood mononuclear cells from the monocytic lineage. M-CSF and RANKL are essential factors for osteoclastogenesis. CSF1R and PU.1 regulate the differentiation of bone marrow cells into osteoclast precursors, which then form multinucleated giant cells by cell-cell fusion. After fusion, proteins specific for osteoclast resorbing activity are expressed. OPG can bind and neutralize RANKL and can negatively regulate both osteoclastogenesis and activation of mature osteoclasts. Molecules are depicted at the stages in which they are predominantly involved. ATP6V0D2 - ATPase H^+ transporting, lysosomal 38kDa V0 subunit d2, CD - cluster of differentiation, CLCN7 - chloride/H^+ antiporter channel 7, CSF1R - colony-stimulating factor 1 receptor, DC-STAMP - dendritic cell-specific transmembrane protein, ERK - extracellular-signal-regulated kinases, JNK - c-Jun N-terminal kinase, MAP2K6 - mitogen-activated protein kinase 6, M-CSF - macrophage colony stimulating factor, MITF - microphthalmia-associated transcription factor, NFATc1 - nuclear factor of activated T cell c1, NF-κB - nuclear factor-κB, OPG - osteoprotegerin, RANK - receptor activator of nuclear factor-κB, RANKL - receptor activator of nuclear factor-κB ligand, Tfe3 - transcription factor E3, TRAF - tumour necrosis factor receptor-associated factor, TRAP - tartrate-resistant acid phosphatase

M-CSF is a hematopoietic growth factor expressed by OB lineage cells involved in the proliferation, differentiation and survival of monocytes, macrophages and bone marrow progenitor cells [35]. Commitment of cells to the myeloid lineage is determined through expression of the transcription factor PU.1. In early stages of hematopoietic stem cell commitment, PU.1 and a heterodimeric complex, formed by microphthalmia-associated transcription factor (MITF) and transcription factor E3 (Tfe3), stimulate the expression of colony-stimulating factor 1 receptor (CSF1R, also called M-CSF receptor), a tyrosine kinase receptor that is the receptor for M-CSF [32, 34]. Mice deficient in M-CSF (op/op mouse) are characterized by osteoclast-deficient osteopetrosis, a disease characterized by

increased bone mass, due to lack of bone resorption, and increased fracture risk, showing the importance of this molecule to osteoclast differentiation [36]. M-CSF interacts with CSF1R expressed on OC precursors, which is essential for their proliferation and survival and stimulates surface expression of RANK [37, 38].

RANKL is a member of the tumour necrosis factor (TNF) superfamily. It is expressed by bone marrow stromal cells, OBs, osteocytes, chondrocytes and immune system cells and interacts with its receptor RANK at the cell surface of OC precursors to induce OC differentiation [39, 40]. RANKL exists both as soluble and membrane-bound forms [3]. The soluble form (sRANKL) corresponds to the c-terminal part of membranous RANKL that may be produced either directly by the stromal cells or OBs through alternative splicing followed by secretion to the extra-cellular medium, or by proteolytic cleavage of membranous RANKL by TNF-alpha converting enzyme (TACE) [41]. Membrane-bound RANKL is expressed at the cell surface of OBs, bone marrow stromal cells, fibroblasts, mammary epithelial cells and activated immune system cells [37]. RANKL binds to its receptor RANK, a member of the TNF receptor superfamily present on OC precursors and mature OCs and stimulates OC differentiation [41]. RANKL also binds to another TNF receptor family member, osteoprotegerin (OPG), which is produced by OBs, bone marrow stromal cells and immune system cells [37, 42]. When bound to RANKL, OPG prevents its binding to RANK and thus inhibits the biological activity of RANKL, being the major negative regulator of bone resorption [43, 44]. $OPG^{-/-}$ mouse exhibit a decrease in total bone density with severe trabecular and cortical bone porosity, thinning of the skull, and a high incidence of fractures [45] demonstrating the importance of the RANKL/OPG ratio in OC differentiation and its bone-resorptive function .

RANK is a type I transmembrane receptor protein expressed primarily on the cells of the monocyte/macrophage lineage including OC precursors and dendritic cells but also on B and T cells and neutrophils [46-48]. $RANK^{-/-}$ mice lack osteoclasts and have a profound defect in bone resorption and remodelling, resulting in osteopetrosis [49, 50]. Although this model showed normal commitment, differentiation and function of dendritic cells and macrophages it had a marked deficiency of B cells in the spleen and lacked all peripheral non mucosal-associated lymph nodes [50]. Concordantly, $OPGL^{-/-}$ mouse was shown to have impaired osteoclast formation as well as defects of thymocytes and B-cell precursors, suggesting the importance of the RANK/RANKL signaling in the immune system [48, 50].

RANK lacks intrinsic kinase activity [32, 34]. It transduces signals by recruiting adaptor molecules such as the TNF receptor-associated factor (TRAF) family of proteins,

particularly TRAFs 1, 2, 3, 5 and 6, which function as adapter proteins to recruit protein kinases [41]. TRAF6$^{-/-}$ mice show impaired interleukin (IL)-1, cluster of differentiation (CD)40 and lipopolysaccharide (LPS) signalling, as well as severe osteopetrosis and deficiency in osteoclast formation indicating that TRAF6 plays an essential function in OCs [51, 52]. TRAF6 activation leads to NF-κB, c-Jun N-terminal kinase (JNK) and the transcription factors activator protein-1 (AP-1), c-myc and nuclear factor of activated T cell c1 (NFATc1) signalling to induce OC formation, Src and mitogen-activated protein kinase 6 (MAP2K6)/p38/MITF to mediate OC resorption and Src as well as extracellular-signal-regulated kinases (ERK) to mediate OC survival [34, 41, 43, 53, 54]. The AP-1 heterodimeric transcription factor complexes are comprised of members of the Fos-related AP1 transcription factor (FRA), Fos, Jun and activating transcription factor (ATF) families and have important roles as regulators of bone development. FRA-2 is a member of the Fos family of transcription factors that regulates the size and survival of OCs [55-57]. Both AP-1 and NF-κB binding sites are present within the promoter region of the NFATc1 gene and NFATc1-deficient mouse embryonic stem cells fail to differentiate to OC, suggesting that NFATc1 is the master transcription factor in osteoclastogenesis [58].

OC precursors stimulation leads to expression of genes coding for molecules required for the fusion of mononuclear OC precursors, such as dendritic cell specific transmembrane protein (DC-STAMP) and the ATPase H^+ transporting, lysosomal 38kDa V0 subunit d2, (ATP6V0D2) [34, 59-62]. Fusion of OC precursors can be divided into 3 steps: firstly, membrane lipid rafts recruit adhesion molecules and align them on the opposing membranes of neighbour cells. Then, the membrane adhesion molecules interact with each other and induce actin rearrangement and an intracellular signal transduction, which is important to fusion and differentiation. The opposing membranes get closer during this interaction. Finally, the force generated by actin polymerization pushes the phospholipid bilayers on opposing membranes into direct contact and results in the formation of a fusion pore. The force generated by actin polymerization is also essential for the expansion of fusion pores and for total cytoplasmic fusion [63, 64].

The molecules participating in the fusion of OC precursors can be divided into RANKL-dependent and independent molecules. CD47, CD9/CD81, ATP6V0D2, macrophage fusion receptor (MFR) and DC-STAMP are regulated by RANK-RANKL pathway. On the other hand, CD44 and triggering receptor expressed on myeloid Cells 2 (TREM2) are important fusogenic molecules, which are not regulated by RANKL [59, 65-67].

A study showed that in DC-STAMP-deficient cells, the expression of osteoclast markers and transcription factors including NFATc1 and RANK are induced under the stimulation of M-CSF and RANKL. In DC-STAMP-deficient mice the number of OC precursors is the same as in wild-type, however, multinucleation does not occur and the bone resorbing area of DC-STAMP-deficient OC is smaller than that of wild-type OC, indicating that DC-STAMP is crucial for OC fusion [68].

After fusion, the OC matures and polarizes with the resorption area towards the bone and the opposite area towards the vascular stream [69, 70]. OCs have an increased number of mitochondria organized near the bone surface and their nuclei are in the opposite region surrounded by endoplasmic reticulum and Golgi's stacks [71, 72]. Mature OCs express high levels of tartrate-resistant acid phosphatase (TRAP), which has been widely used as a cytochemical marker of OCs and their precursors [73] and several other genes that regulate their resorptive ability including those encoding the chloride/H^+ antiporter channel CLC7 and cathepsin K [32, 34].

Bone resorption

Bone resorption is a complex and specific process that occurs in three stages: (i) OC attachment to bone matrix and cell polarization, (ii) resorption, depicted in Figure 2, and (iii) cessation of resorption.

OC attachment to bone surface is mainly dependent on integrins, which are a superfamily of transmembrane proteins composed of an α and a β chain that can transduce signals bidirectionally [74]. In the OC, the most important integrin is the vitronectin receptor (αvβ3 integrin) [32] that recognizes the RGD motif of bone matrix proteins such as OPN [53]. αvβ3 integrin mediates not only OC motility but also adhesion to the bone matrix, which is required for polarization of the resorptive machinery and formation of an isolated, acidified microenvironment [17]. Attachment to the bone surface by integrins results in recruitment of Src tyrosine kinase and to the activation of Src-dependent signalling pathways that allows the cell to spread and form actin rings. [34, 75]. After attachment, and consequently polarization, OCs organize their actin cytoskeleton into an actin ring in order to form the sealing zone [76]. At this moment, podosomes are formed. These are highly dynamic structures formed by a core of densely packed actin filaments and F-actin-associated proteins and are of major importance for bone resorption and OC motility [32, 76-79]. The membrane adjacent to the sealing zone becomes a highly convoluted ruffled membrane, due to podosome formation and to the fusion of acidic vesicles of the

endocytic/lysosomal pathway that transport the resorption machinery such as cathepsin K, TRAP, vacuolar H^+ATPase and the Cl^-/H^+ antiporter channel CLCN7 to the plasma membrane in direct contact with bone [32, 34, 75, 79].

The acidification process of the resorptive lacunae is initiated by carbonic anhydrase (CA), which generates protons (H^+) and HCO_3^- ions. Protons are secreted into the resorption lacuna by the vacuolar ATPase and the CLCN7 charge-coupled to the ATPase acts as a chloride-proton antiporter (transporting Cl^- ions into the resorptive lacuna) [77, 80]. Hence, OCs need both functional H^+ATPase and CLCN7 in order to acidify the underlying resorption lacuna [81]. Intracellular pH is maintained by a Cl^-/HCO_3^- exchanger on the OC's antiresorptive surface [53]. H^+ and Cl^- ions pass through the ruffled membrane and form hydrochloric acid (HCl) to dissolve the mineral component of bone [77, 82]. Acidification of the resorption lacuna precedes collagen degradation by a few hours, demineralizing the bone matrix and exposing the organic components of bone [83]. After acidification, lysosomal enzymes are released into the lacuna [53, 72].

TRAP is a soluble acid resistant phosphatase secreted by the OC that plays a role in bone resorption. TRAP knockout (KO) mouse have deformities in the long bones and axial skeleton and increased mineralization reflecting a mild osteopetrosis caused by reduced osteoclast activity. These mice also display impaired macrophage function, with abnormal immunomodulatory cytokine responses and increased superoxide formation and nitrite production in stimulated macrophages [84]. Although TRAP's physiological substrates have not been identified yet, the candidates are phosphorylated tyrosine, sugar-phosphates as adenosine tri-phosphate (ATP), mannose-6-phosphates and bone matrix phosphoproteins, such as phosphorylated OPN [72, 85, 86]. Another function of TRAP is to generate reactive oxygen species (ROS) that are capable of destroying collagen and other matrix proteins such as OPN, OCN and BSP [73, 79].

After removal of the inorganic bone phase and exposure of the matrix proteins, pro-cathepsin K is activated. Cathepsin K is secreted by OCs to degrade several bone matrix proteins, among which are included type I collagen and OPN among others.

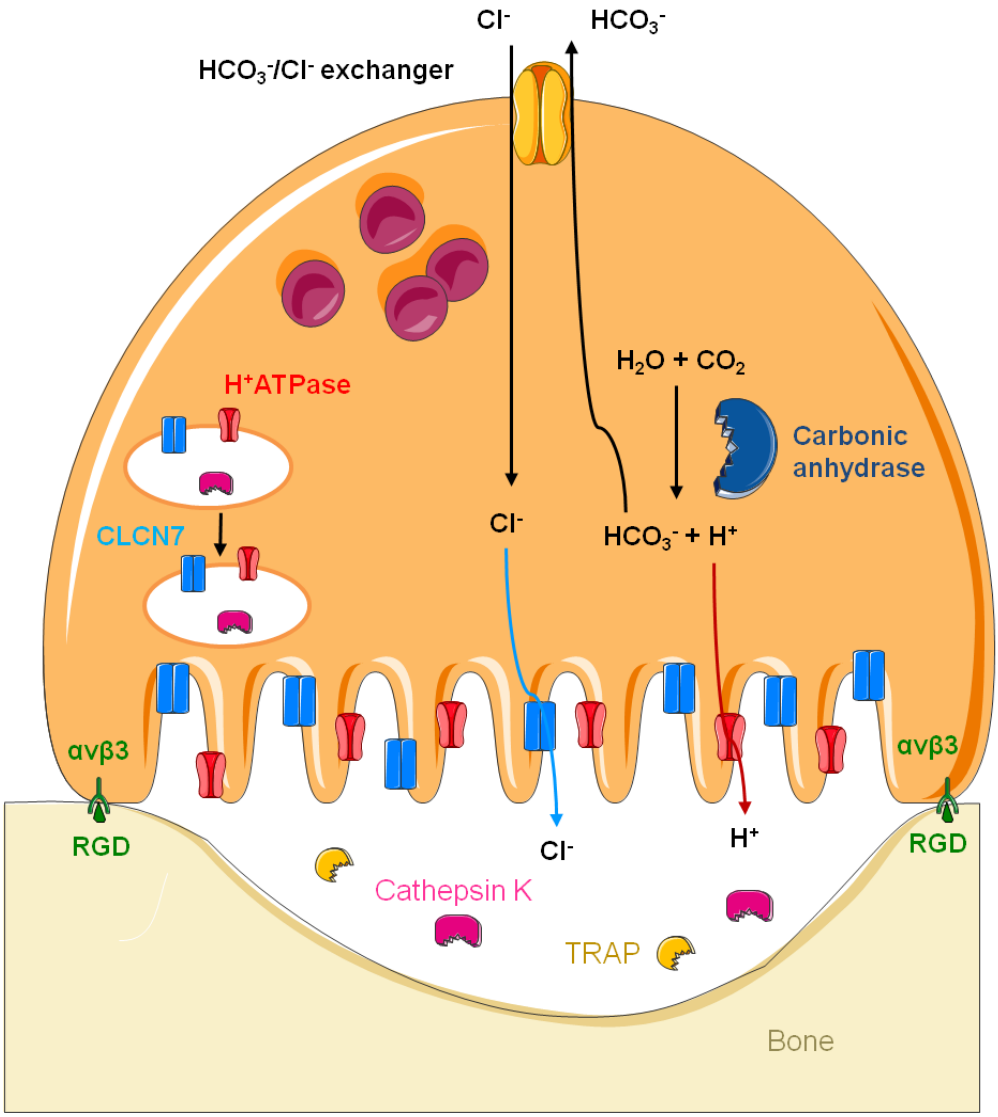

Figure 2 - Mechanisms of osteoclastic bone resorption. Bone resorbing osteoclasts adhere to bone throught αvβ3 vitronectin receptor binding to RGD residues and form sealing zones. The resorbing area under the ruffled border is acidic and several transport systems including the H^+ATPase proton pump, Cl^-/HCO_3^- exchanger and chloride channel are responsible for the acidification of this lacunae. ATP - adenosine triphosphate, CLCN7 - chloride/H+ antiporter channel 7, RGD - amino acid sequence - arginin-glycin-aspartic acid, TRAP - tartrate resistant acid phosphatase.

Cathepsin $K^{-/-}$ mouse display an osteopetrotic phenotype with excessive trabeculation of the bone-marrow space and abnormal joint morphology. Cathepsin K deficient OCs showed altered morphology with poorly defined resorptive surfaces and a large demineralized matrix portions with undigested collagen fibrils. Moreover, the resorptive activity of cathepsin K-deficient OCs was severely impaired *in vitro* [87, 88].

It is not clear where activation of pro-cathepsin K into cathepsin K occurs. One study has shown that it occurs intracellularly, before secretion into the resorption lacunae and the

onset of bone resorption [89]. However, the most accepted theory is that pro-cathepsin K activation occurs in the acidic environment created in the resorption lacunae. In this microenvironment, the low pH induces a conformational change of the pro-enzyme and makes the pro-peptide more accessible to cleavage [90, 91]. Cathepsin K degrades type I collagen in its non-collagenous termini (N- and C-telopeptide regions) and releases cross-linked N- and C-telopeptides (NTX-I and CTX-I), which can be detected in urine and serum by immunoassays and are currently used to assess bone turnover [92, 93]. Once degraded, bone matrix products like CTX-I and calcium ions (Ca^{2+}), are internalized by endocytosis in the central area of the ruffled membrane and are then transported to the functional secretory domain, opposed to the resorbing area, to be secreted, in a process of transcytosis [72, 94, 95]. This mechanism allows OCs to remove large amounts of matrix-degradation products without losing their tight attachment to the underlying bone [32, 96].

The signal by which the OC stops its resorbing activity is unknown. Several factors have been proposed to act as a stop signal, such as calcitonin [97], estrogen and transforming growth factor-β (TGF-β) [98, 99] or extracellular calcium concentration [100]. Calcitonin depresses serum calcium and this was associated with retraction of podosomes and ruffled membrane *in vitro* [101, 102]. Estrogen promotes osteoclast apoptosis by stimulating TGF-β release from OBs [98]. TGF-β has a direct and contradictory action on osteoclasts, because on one hand it limits the proliferation and fusion of osteoclast precursors, and on the other hand enhances RANKL-mediated differentiation [103, 104]. In addition, TGF-β is a potent inducer of osteoclast apoptosis through the up-regulation of Bim, a member of the pro-apoptotic Bcl2 family [98, 105]. Calcium is also a very important regulator of bone resorption. When the concentration of calcium released from the bone surface rises, it leads to disruption of cytoskeletal structures and, as a consequence, inhibits resorption [100]. Once the calcium concentration is diluted, the OC repolarizes and assembles a new sealing zone. OCs are highly motile cells and are able to migrate from one site of resorption to a new one. Their capacity to achieve this function is only possible by quickly disassembling and reassembling their podosomes. Optimal bone resorption depends upon these cycles of attachment, sealing zone formation and migration [32, 72].

OCs are more than resorptive cells, they not only regulate bone homeostasis through bone remodelling, but also by regulating calcemia and phosphatemia by mobilization and exchange of ions [106, 107]. OCs can interact with a multitude of other cell types including immune system cells, hematopoietic cells and OBs [72, 108]. They also contribute to the formation of the hematopoietic niche and to angiogenesis by releasing

growth factors from the bone matrix like C-X-C motif chemokine 12 (CXCL12) [109] and by directly or indirectly (by the release of TGF-β) increase the expression of vascular endothelial growth factor (VEGF) [110, 111].

Osteoblasts

Osteoblasts are mononuclear, non-terminally differentiated, specialized cells that derive from the mesenchymal stem cell lineage and have the ability to secrete bone matrix where hydroxyapatite crystals deposit [112-114]. The differentiation of a mesenchymal stem cell into a functional OB requires the presence of three transcription factors, runt-related transcription factor 2 (Runx2, also known as core-binding factor subunit alpha-1 - Cbfa1), osterix (Osx) and β-catenin [115]. Runx2 is expressed at early phases of osteoblastogenesis and is responsible for mesenchymal cell commitment [116]. In $Runx2^{-/-}$ mouse both intramembranous and endochondral ossification were completely blocked due to the maturational arrest of osteoblasts [117, 118]. These mice also have no Osx expression [119]. Osx is determinant in lineage commitment towards osteoblast differentiation because it is responsible for the transcription of BSP, OCN and the increase in type I collagen expression [119]. Osteoblast differentiation and function, and therefore bone formation, is under the control of bone morphogenetic proteins (BMPs) and Wingless (WNT) signalling pathways [30, 115].

BMPs belong to the TGF-β superfamily and are involved in the early steps of osteoblastogenesis [8]. It has been described that BMP-2, BMP-4 and BMP-7 are able to induce immature cells to differentiate to osteoblasts [120] and specifically that BMP-7 induces the expression of Runx2 indicating that this gene is a target of BMP signalling in early osteoblast differentiation [115, 121].

The canonical Wnt/β-catenin pathway is essential for OB differentiation during skeletal formation and development and continues to be important in mature OBs and cell death [122, 123]. In the absence of Wnt proteins, glycogen synthase kinase-3β (GSK-3β) phosphorylates β-catenin, which is degraded, and the OB signalling cascade is blocked, so the mesenchymal stem cells (MSCs) become chondrocytes or adipocytes [123, 124]. When Wnt proteins are present they bind to the frizzled receptor and to low-density lipoprotein receptor-related protein 5/6 (LPR5/LRP6), activating the signalling cascade [125]. These receptors transduce a signal to a complex formed by dishevelled (Dsh), GSK-3β, axin and adenomatous polyposis coli (APC), which promotes the phosphorylation and inhibition of GSK-3β [126, 127]. As a result, β-catenin accumulates in the cytoplasm and translocates to

the nucleus, where it interacts with the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) and induces the expression of OB related genes and OPG [126].

Some molecules specifically block the Wnt pathway such as secreted Frizzled related proteins (sFRPs), Wnt inhibitory factor (WIF), dickkopf-related protein 1 (DKK1) and sclerostin (SOST) [125, 128-130].

DKK-1 is a soluble inhibitor of Wnt pathway produced by osteocytes and OBs [122]. It interacts with LRP5/6 and transmembrane proteins kremen1 and 2, leading to the internalization of this complex and degradation by the proteasome [131]. Its overexpression leads to inhibition of OB proliferation and impaired mineralization [130] and its blockade leads to increase in bone mass [132-134].

Another inhibitor of the Wnt pathway is sclerostin a secreted protein mainly expressed by osteocytes [135]. This protein is secreted in response to mechanical stimuli, arresting bone formation, as shown in both animal model and human disease [136-140]. Like DKK1, SOST inhibits Wnt/β-catenin pathway by binding to LRP5/6 co-receptor; however, it binds to a different region of LRP5/6 and it does not mediate receptor internalization [122, 141]. In vitro, sclerostin has been shown to inhibit OB proliferation, to impair mineralization by OBs and to stimulate OB apoptosis by interfering with both Wnt and bone morphogenetic protein (BMP) signalling [30, 115, 135, 142, 143].

Following lineage commitment and expression of type I collagen, OB progenitors undergo a proliferative stage where they acquire ALP activity. Subsequently, they exit the proliferative stage and begin to express genes encoding matrix proteins such as BSP, OCN and more type I collagen as they start to produce and mature the extracellular matrix [115]. Finally, osteoprogenitors express genes of proteins involved in mineralization of the extracellular matrix such as OCN and OPN [144, 145]. After carboxylation, OCN attracts calcium ions and incorporates them into hydroxyapatite crystals, thus mineralizing the collagen scaffold [146, 147].

OBs have many functions in the human bone: they are responsible for bone matrix synthesis and mineralization and are important regulators of osteoclastogenesis as they express several proteins that are crucial for OC differentiation like M-CSF, RANKL and OPG. More recently, OBs have been described to have an important role in the endocrine system by production of OCN that regulates adipose tissue insulin sensitivity [148], promotes testosterone synthesis [149] and also crosses the blood-brain barrier, binding to neurons and protecting them from apoptosis [115, 150]. In addition, it was recently shown that OBs have a role in the hematopoietic niche by supporting in vivo Notch1 activation

and increasing the number of haematopoietic stem cells [151] and that β-catenin might be associated with acute myeloid leukemia [115, 152].

At the end of the bone formation phase OBs have one of three possible fates: they can be embedded in the matrix and differentiate to osteocytes, they can become bone lining cells or they can undergo apoptosis [115, 153-156].

Osteocytes

When osteoblasts become entrapped in the bone matrix a dramatic shift in both shape and function occurs. From a cuboidal cell specialized for active secretion of extracellular matrix to a "dendritic" cell with a small cell body and numerous long, slender processes that connect to their neighbours. Osteocytes are the most common cells in bone, as well as the most long-lived, as their lifespan reaches 25 years [115, 157]. The dendrites are used to communicate with each other and with other cells such as OBs and OC progenitors through an extensive system of interconnecting canaliculi [158-161]. The communication is also achieved by the interstitial fluid that flows through the canaliculi and is essential in coordinating the response of bone to mechanical and biological signals [162].

Formation of osteocyte processes begins before the bone matrix mineralizes. The specific mechanisms have yet to be fully elucidated *in vivo*. However, they appear to be dynamic and dependant on the type of bone in which the OB/osteocyte is in [163-166]. There are currently four different hypothesis by which osteoblasts could become entrapped in bone matrix as osteocytes: 1) Osteoblasts secrete matrix in all directions. 2) Each osteoblast is polarized in a different direction but still secretes matrix in one direction only. 3) One generation of osteoblasts buries the next generation. 4) The osteoblast to be embedded slows down its matrix production compared to neighbouring osteoblasts; each of these mechanisms may occur in different bone types [161].

Osteocytes have diverse functions in bone homeostasis. They are mechanosensors that translate mechanical stimuli into biochemical signals, which in turn regulates bone turnover [167]. When responding to mechanical loading osteocytes can produce two important inhibitors of OB function, SOST [135] and DKK1 [130, 168], but might also reduce the expression of SOST through a negative feedback loop [169]. Osteocytes can also regulate calcium release. In the early 70’s, Ramp and Neuman hypothesized that osteocytic osteolysis accounted for more than 90% of total calcium release, through the acidification of the osteocyte lacuna [170, 171]. It has also been shown that some

osteocytes are positive for TRAP, thus hypothesizing that osteocyte TRAP activity could be stimulated by the same factors that stimulate osteoclast activity [115, 172, 173].

Osteocytes are able to sense bone micro fractures, thereby signalling the need for repair [168, 172]. The death of osteocytes by apoptosis signals the presence of damage on its location and is considered the initiation of targeted remodelling [115, 155]. When apoptotic, osteocytes express higher levels of RANKL, TNF and other factors that travel through the lacuno-canalicular system to the bone surface and are sensed by the progenitor cells, thereby initiating a cycle of remodelling [115].

Bone remodelling

Bone remodelling is a dynamic process that renews and substitutes ischemic or micro fractured bone; regulates calcium homeostasis and renews old bone. In this process bone undergoes continuous destruction and formation at several sites (basic multicellular units, BMUs) throughout the skeleton in response to mechanical and metabolic effects [174]. It relies on the accurate balance between bone resorption by OCs and bone formation by OBs, whose functions must be tightly coupled, not only quantitatively but also in time and space [8]. In trabecular bone, remodelling occurs on the surface of trabeculae and lasts about 200 days in normal bone [175-177].

Bone remodelling is initiated by the osteocyte when it recognizes a specific area to be replaced. Signals like chemokines and growth factors arise to the bone surface through the canaliculi and induce migration of OC and OB precursors. When resorption is needed at a particular site, bone lining cells detach to form a raised canopy where the BMU can repair the damaged area. This is called the bone remodelling compartment (BRC) [178]. Inside the BRC, OC precursors enter through bone capillaries. These precursors will differentiate to OCs due to the expression of M-CSF and RANKL by bone lining cells, apoptotic osteocytes and early OB progenitors [179]. OCs are then formed and resorb bone in the affected area. An essential requirement for balanced remodelling is that the amount resorbed is replaced by an equal amount of new bone. This is achieved by several coupling factors inside the BMU that coordinate the OC/OB activity. Inside the BMU, OCs and OBs communicate not only with each other, but also with cells from the vascular and immune systems [174]. After resorption, bone surface remains covered with undigested demineralized collagen matrix and the cells responsible for the removal of matrix debris act during this phase cleaning the lacunae and preparing for a cycle of bone formation [180, 181].

It is now well established that during bone resorption growth factors including TGF-β and insulin growth factor-1 (IGF-1) are released from bone matrix and stimulate OB recruitment and activation. OCs can also secrete proteins that will lead to OB activation like cardiotrophin-1 (CT-1), BMP-6, Wnt10b and sphingosine-1-phosphate (S1P) [174, 182]. Coupling is also achieved by direct communication between OB and OC, not only through M-CSF and RANKL/OPG, but also through a class of surface bi-directional signalling proteins, the ephrins, being the most studied the ephrinB2/EphB4 binding, which is involved in OB differentiation and activity [183].

After bone resorption, OBs need to mature and deposit new bone matrix, completing the remodelling process. Collagen type I is the primary organic component of bone and non-collagenous proteins add the remaining organic material. Ultimately, hydroxyapatite crystals are incorporated into this newly deposited osteon [9]. The signal that terminates the remodelling process is still unknown, although it is believed that when OBs become embedded in the mineralized matrix and differentiate to osteocytes, SOST expression increases, bringing an end to the remodelling cycle [174, 180].

The correct balance between bone deposition and resorption is crucial for the proper maintenance of bone mass and loss of this coupling mechanism is the starting point for several skeletal pathologies, such as osteoporosis, characterized by low bone mineral density (BMD) and increased resorption, or osteopetrosis characterized by excess bone formation that leads to high BMD [184, 185].

Bone turnover markers

Biochemical markers are proteins usually measured in blood or urine used to assess bone metabolism. These are very important in clinical practice as they allow the understanding of bone remodeling status in a non-invasive way. They can be classified as markers of bone resorption that may be associated with OC function or may reflect bone matrix degradation and, on the other hand, markers of bone formation that are associated with OB function and with type I collagen assembly [186, 187].

OCN, bone specific ALP and the C and N-propeptide of type I procollagen (P1CP and P1NP, respectively) are examples of the most relevant bone formation markers [188]. OCN is a small non-collagenous protein synthesized by osteoblasts and although it is a sensitive marker of bone formation, the use of its measurement in clinical practice is limited by assay variability, sample instability and high biological variability [188, 189]. Bone

specific ALP is produced by osteoblasts and its production is correlated positively with bone formation rate [190-192]. Its detection in clinical samples relies on highly variable detection methods that can be inaccurate, however this is still one of the major bone turnover markers to be used in Paget's disease, chronic kidney disease and in cancer patients [193-195]. During bone formation, procollagen is cleaved at the N- and C-terminal ends so P1CP and P1NP reflect the rate of new bone formation [196]. Both P1CP and P1NP can be measured in serum, however the analysis of P1NP is more extensively described in the literature than that of P1CP [188]. Circulating P1NP and P1CP concentrations have been found to be directly proportional to the amount of new collagen matrix being formed and subsequently, newly mineralized bone. Serum P1CP has been shown to be useful indicator for predicting bone metastases and response to treatment in solid prostate tumours and kidney disease [197-201]. Serum P1NP levels are frequently used to monitor bone formation in humans [202].

Bone resorption markers include TRAP, collagen cross-link molecules (pyridinoline (PYD) and deoxypyridinoline (DPD)), cross-linked telopeptides of collagen I, hydroxyproline and hydroxylysine-glycosides, bone sialoprotein, cathepsin K and RANKL/OPG [188].

During bone resorption, osteoclasts secrete TRACP5b (the osteoclast specific isoform of TRAP), which produces reactive oxygen species to digest bone degradation products in the microenvironment of the bone matrix and it is used as an index of osteoclast activity and numbers [203]. PYD and DPD are molecules that mechanically stabilize the collagen molecule, by crosslinking between individual collagen peptides. Because DPD is almost solely found in bone and PYD is also expressed in cartilage, DPD is a more specific and sensitive marker than PYD [204]. Increased production of collagen cross-links is seen in metabolic bone diseases associated with increased bone turnover [205].

Telopeptides of type I collagen are the most extensively studied and used bone resorption markers. There are two sites that originate the telopeptides: the N-terminal and the C-terminal [188]. The N-terminal originates the N-terminal telopetide (NTX-I). On the C-telopeptide end, two fragments have been characterized and include the cross-linked carboxyterminal telopeptide of type I collagen (ICTP), a cross-link-containing collagen peptide originally isolated by trypsin digestion of human bone collagen, and C-terminal crosslinked telopeptide of type I collagen (CTX-I) [206]. CTX-I and ICTP respond differently according to the clinical situations and treatments. Serum and urinary CTX-I levels are increased in postmenopausal women compared with premenopausal controls.

These changes in CTX-I levels contrast with the only slight and non-significant changes of serum ICTP in the same conditions [207, 208]. Serum ICTP has however shown to be a valuable index of bone turnover in other pathological situations, including patients with bone metastases of breast, prostate and lung cancer and multiple myeloma [209-211]. These two products are generated by different enzymes: while CTX-I is a product of cathepsin K degradation of collagen, ICTP is a product of matrix metalloproteinases (MMPs) MMP-2, -9, -13, or -14 collagen degradation [202, 206].

BSP, cathepsin K and the ratio RANKL/OPG are potentially useful markers of bone resorption and have been used in both clinical and non-clinical research [212-215], however further studies are required for commercial use, mainly due to inconsistent results and a further need of improvement in assay methods [188].

Bone and inflammation

Bone and the immune system share several regulators and players. This connection is so strong that, in 2009, the term 'osteoimmunology' was used for the first time to describe this tight relationship [216].

Immune system cells

The hematopoietic stem cell (HSC) niche is a specific environment in the bone marrow. Bone and bone marrow are now considered a single organ, with a hard bony cortex and a medullary parenchymal core with bony trabeculae, in which hemato-lymphopoiesis progresses under the control of bone cells, mainly OBs, as OCs importance is still under investigation [72, 217]. However, communication between immune hematopoietic progenitors and bone cells is not a one-way road and immune system cells also influence how OBs and OCs function not only in the normal, but also in the disease state.

Neutrophils

Neutrophils are the predominant immune cell population in human blood. These cells are derived from hematopoietic precursors on the bone marrow and are released into circulation to patrol the blood stream and protect the human body from pathogens being indispensable for fighting bacterial and fungal infections. Neutrophils express several pro-inflammatory and pro-osteoclastogenic cytokines like IL-1β, IL-6, IL-17A, immunoregulatory cytokines like TGF-β, interferon-gamma (IFNγ) and RANKL and

chemokines like CXCL12 [218, 219]. Neutrophils have been shown to express surface RANKL when activated by toll like receptor (TLR)-2 and 4 to stimulate osteoclastogenesis [220, 221]. In a 2012 report it was also shown that neutrophils express RANK and that they are able to migrate through a RANKL gradient, however, no evidence of further activation after RANKL binding was studied [222]. More recently it was shown that these cells can transdifferentiate to monocytes, the OC precursors [223]. On the other hand, neutrophils can prevent OB differentiation either by inhibiting stromal cell-derived factor 1 (SDF-1) expression on MSCs or by inducing OB progenitors' apoptosis through reactive oxygen species (ROS) production [224].

Dendritic cells, monocytes and macrophages

OCs share a common myeloid progenitor with monocytes, macrophages and dendritic cells (DCs), directly linking bone to the innate immune system [225-227]. Both dendritic cells and monocytes have at their surface the major histocompatibility complexes (MHC - human leukocyte antigen (HLA)) that present antigens to T lymphocytes.

DCs consist in two main subtypes, plasmacytoid (pDCs) and conventional (cDCs). The major function of pDCs is in response to virus and virus derived nucleic acids. On the other hand, cDCs are professional antigen presenting cells (APCs), whose major function is to process and present antigens to naive T lymphocytes, linking the innate and adaptive immune system [228]. Both pDCs and cDCs are widely distributed in the mucosa and in the spleen, but in the context of inflammatory diseases pDCs, cDCs and their monocytic precursors are recruited into peripheral tissues and can act as OC precursors [77, 229]. It has already been shown that DC-derived OCs are able to resorb bone [230, 231]. In addition DCs indirectly increase the bone turnover by activating T-cells to express IFNγ, RANKL and IL-17 [230]. Interestingly, activation of T lymphocytes diverts the monocyte progenitor towards DC differentiation by down-regulation of RANK and c-Fos expression through IL-3 secretion [232-234].

Monocytes are originated from hematopoietic stem cells in the bone marrow. They constitute 5 to 10% of the circulating leukocytes and have a circulating half-life of 3 days in humans [235-237]. Monocytes are a heterogeneous population in terms of surface markers, phagocytic capacity and differentiation potential. Circulating monocytes differentiate to a multitude of cells including macrophages, DCs, OCs, microglia in the central nervous system, Kupfer cells in the liver, endothelial cells and to a lower extent into cardio-myogenic and neuronal precursors [238-241]. It is currently accepted that there

are three sub-populations of monocytes in humans, based on their expression of CD14 and CD16 surface markers [238, 240]. The classical subset, $CD14^{bright}CD16^{-}$, accounts for about 85% of monocytes. These cells are phagocytic and are considered to be OCs precursors. The classical subset is characterized by CD62L, CXCR1, CXCR2 and C-C chemokine receptor type 2 (CCR2) expression and secretion of IL-6, IL-8, IL-10, granulocyte colony stimulating factor (G-CSF) and chemokine (C-C motif) ligand 5 (CCL5). The intermediate subset, $CD14^{bright}CD16^{+}$, is the most recently described, accounting for only 5% of monocytes [242]. This subset is considered to be the antigen presenting subset due to the surface expression of HLA-DR and CD105 (endoglin, an important co-receptor of TGF-β [243, 244]) and is responsible for ROS production. The intermediate subset is characterized by secretion of both IL-6 and IL-8. The non-classical subset, $CD14^{dim}CD16^{+}$, accounts for 10% of monocytes and is involved in cytokine production (TNF, IL-1β, IL-6 and IL-8) and T-cell activation due to the surface expression of CD43, a transmembrane protein involved in the ligand-receptor T cell complex activation [240, 245]. These three subpopulations have been showed to differentiate to OC [246].

Macrophages are immune cells that are present in the tissues and exhibit tissue-specific characteristics. In bone there are at least two types of tissue macrophages. Osteoclasts ($Calcitonin^{+}TRAP^{+}$) are considered a specialized type of macrophage that is responsible for bone resorption. On the other hand, $TRAP^{-}CD169^{+}$ macrophages, also called 'osteomacs' are involved in the removal of bone debris from the bone surface after bone resorption, regulate osteoblast function and are also involved in the maintenance of the erythropoietic niche in the bone marrow [179, 247-249].

The ability of the myeloid-lineage cells (DCs, monocytes and macrophages) to specifically regulate OBs has also been shown. Osteomacs have been shown to form a canopy over mature OBs at sites of bone formation, as well as being in close association with bone lining cells [179]. Monocyte/macrophage lineage cells enhance osteogenic differentiation and activity from MSCs through secretion of BMP-2 and oncostatin M (OSM) [228, 250-252].

T lymphocytes

T lymphocytes play a central role in cell-mediated immunity. T lymphocytes mature in the thymus and each subset of T lymphocytes has a distinct function.

Activated T lymphocytes can support osteoclastogenesis through the production of cytokines such as IL-1, IL-6 and IL-17A since they act on OBs to induce RANKL expression [253, 254]. There is also evidence that TNF contributes to gene expression of specific OC proteins and that it directly activates OC differentiation from precursors through cross activation of the NF-κB pathway or JNK signalling cascades [255]. On the other hand, resting T lymphocytes have been shown to inhibit OC differentiation by balancing TNF and IFNγ expression [48, 256, 257]. While TNF has been shown to up-regulate RANKL expression on synovial fibroblasts, IFNγ has been shown to inhibit osteoclastogenesis [258-260]. T cell derived IFNγ was found to potently inhibit RANKL signalling through down-regulation of TRAF6 in OC precursors [259, 261-264]. RANKL-deficient mice not only show a severe osteopetrotic phenotype but also defect in B and T cell differentiation and have no lymph nodes [48]. Studies have shown that RANKL is not only expressed by OBs and fibroblasts but also by activated T and natural killer (NK) cells [48, 256, 265, 266]. In oestrogen deficient mice the formation of T helper 17 cells (Th17) cells is associated with downregulation of Foxp3, a marker of regulatory T lymphocytes (Tregs). The resulting increase in IL-17A expression induced bone loss by increasing pro-osteoclastogenic cytokines like RANKL, IL-6 and TNF [267]. Moreover, the balance between Tregs and T effector cells like Th17 and Th1 is regulated by TNF expression [268]. Tregs have been shown to inhibit the formation of OCs through the production of TGF-β and IL-4 [269, 270], which limits bone resorption by inducing OPG expression and suppressing expression of RANKL, RANK, NF-κB, c-Fos, NFATc1 and calcium signalling during OC formation [271].

Recent studies show that IL-17A in synergistic action with TNF can induce ALP activity and increase Runx2 expression in MSC and stromal cells [258, 272, 273]. This effect is dependent on the OB differentiation state since TNF can inhibit OB formation if it acts on the pre-OB stage inhibiting either the Wnt or the BMP pathway [274, 275].T lymphocytes have also been implicated in osteogenic differentiation during fracture repair by the production of IL-17F, stimulating bone healing [276]. These cells also have a role in postmenopausal osteoporosis bone formation induced by intermittent parathyroid hormone (PTH) therapy. Bedi *et al.* showed that silencing PTH receptor specifically on T lymphocytes reduced Wnt10b production by OBs and decreased bone turnover, bone mineral density and trabecular bone volume [277]. However, further studies are needed to address the effects of T lymphocytes on OBs [228]. The context of T cell presentation

determines whether they are pro- or anti-anabolic, but based on these studies the balance appears to favour a role in anabolism [217].

B lymphocytes

B lymphocytes are key players in both innate and adaptive immunity. In addition, they have functions that go beyond the immune system and are involved in tissue repair [278-280]. B cell development occurs within the bone marrow and relies on factors produced not only by stromal cells but also by OBs, such as a proliferation-inducing ligand (APRIL), B cell activating factor (BAFF), CXCL12, stem cell factor (SCF), IL-7, RANKL and OPG [281, 282]. Autologous RANKL expression in early B cell development stages is essential for B cell development and correct bone homeostasis [48, 283]. Moreover, studies associated specific B cell RANKL expression to OC-mediated bone loss after ovariectomy, stressing the importance of these cells on osteoclastogenesis [283]. On the other hand, plasma cells produce the highest concentrations of OPG per cell followed by mature B lymphocytes. In earlier stages of B cell development where RANKL is expressed, OPG is secreted in low amounts [284]. B lymphocytes also express RANK. In $RANK^{-/-}$ mouse, in which the RANK gene was excised at the very early pro-B cell stage, no B cell abnormalities or altered lymphoid organ development were observed indicating that in the early stages of differentiation of hematopoietic stem cells, RANKL engages in an alternative cellular receptor to RANK in pro-B lymphocytes [285]. In 1999 studies with $Pax5^{-/-}$ mouse cells showed that if early B lymphocytes are treated with M-CSF and RANKL they differentiate to TRAP positive multinucleated OC-like cells showing that although from different lineages, there is a common immature hematopoietic precursor shared by B lymphocytes and OCs [281, 286].

B lymphocytes can also indirectly influence bone mass due to their interaction with T lymphocytes and by the production of cytokines. B lymphocytes secrete pro-inflammatory cytokines like IL-1β, IL-6 and TNF, growth factors such as M-CSF, granulocyte-macrophage (GM)-CSF and IL-7 and anti-inflammatory cytokines like TGF-β and IL-10 [287]. B lymphocytes have been shown to inhibit the formation of OCs by the secretion of IFNγ, IL-10 [288] and TGF-β that induces mature OCs apoptosis [289]. However, TGF-β has opposite effects on early OC progenitors, inducing RANK expression and working in synergy with RANKL [290-293]. IL-10, an anti-inflammatory cytokine, which functions to contain inflammation, limits OC formation by inhibiting the expression of c-Fos, c-Jun and NFATc1 in OC precursors [271]. Moreover, there is strong evidence on the role of the OB

lineage on B cell development in the hematopoietic niche, mainly by IL-7 OB production and by sclerostin production by osteocytes [294-297]. However, the role of B lymphocytes in the OB lineage is less understood.

Clear evidences of the interplay between the immune system and osteoclasts are summarized in figure 3. In chronic immune mediated diseases such as rheumatoid arthritis and ankylosing spondylitis the immune system is hyper activated and this poses consequences for bone.

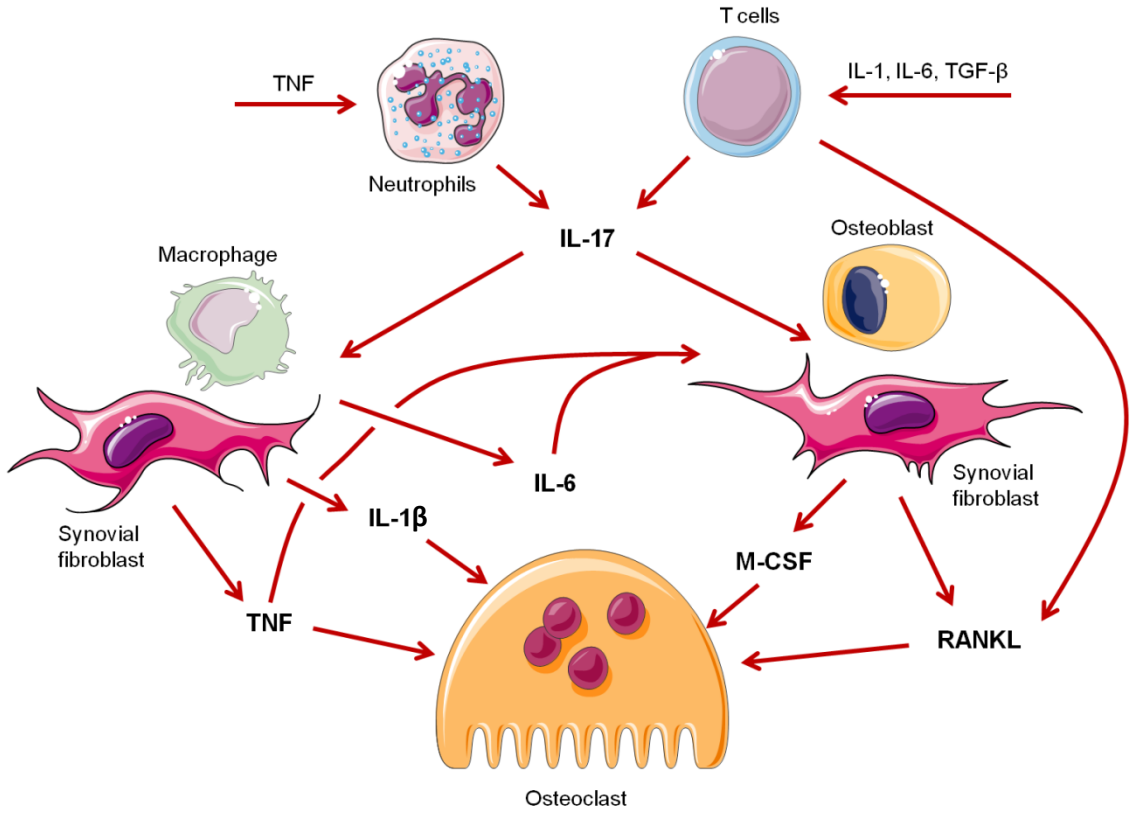

Figure 3 - Cytokines activating bone resorption. TNF, IL-1β, IL-6 and IL-17 upregulate expression of RANKL in osteoblasts, synovial fibroblasts and immune system cells. RANKL mediates differentiation, survival and activation of osteoclasts. TNF and IL-1β promote differentiation and survival of osteoclasts. IL-6 enhances expression of RANKL and contributes to the activation of immune system cells that secrete IL-17 and other cytokines. IL-17 induces the expression of RANKL in osteoblasts, synovial fibroblasts and immune system cells and enhances the secretion of pro-inflammatory cytokines by macrophages.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, immune mediated inflammatory disease that mainly affects the synovium of multiple joints, leading to the progressive destruction of cartilage and bone. This disease is characterized by pain, swelling and destruction of the synovial joints causing functional impairment and a significant increase of both morbidity and mortality [298, 299].

The chronic nature of RA results in personal costs as it is associated with serious disability, reduced quality of life, loss of work capacity and diminished life expectancy [300]. Epidemiological studies have shown that RA affects about 1% of the world population and 75% of RA patients are women [301, 302]. In Portugal it has been reported that the prevalence of RA is 0.7% and it is also more frequent in women than in men [303, 304]. Its incidence is highest among those aged between 30 and 50 years and, due to its chronic nature, the prevalence increases with age, as well as co-morbidities [302, 305].

The predominant symptoms of RA are symmetrical pain, stiffness and swelling of peripheral joints. The clinical course of the disease is variable, ranging from mild to severe and highly destructive arthritis. The analysis of the clinical course and of laboratory and radiologic parameters have defined prognostic factors related to progressive joint destruction, such as the presence of autoantibodies like rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [306-308]. In some patients these markers emerge years before the onset of the disease and probably contribute to its development and chronicity [309-311]. For classification purposes, mainly related to research, patients are classified as having RA based on the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) rheumatoid arthritis classification criteria (see table 1). This classification criteria is based in several variables including the number and type of involved joints, presence of RF and/or ACPA, elevated acute-phase proteins and symptoms duration [312]. The ultimate goals in managing RA are to decrease symptoms as early as possible and to prevent joint damage and loss of function. A prompt diagnosis and an accurate early therapeutic strategy are essential to prevent RA progression and joint erosions, which can occur as early as 4 months after the onset of the disease [313, 314]. Tight disease control and treatment driven by a predefined disease activity target are crucial for an effective long-term management of RA. Thus, it is important to diagnose RA in the earliest possible phase of the disease course.

Table 1 - The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis

	Score
Target population are patients who:	
1) have at least 1 joint with definite clinical synovitis (swelling)*	
2) with the synovitis not better explained by another disease†	
Classification criteria for RA (score-based algorithm)	
Add score of categories A–D.	
A score of ≥ 6/10 is needed for classification of a patient as having definite RA)‡	
A. Joint involvement§	
1 large joint#	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)##	2
4-10 small joints (with or without involvement of large joints)	3
> 10 joints (at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)††	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)‡‡	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms§§	
< 6 weeks	0
≥ 6 weeks	1

* The criteria are aimed at classification of newly presenting patients; † Differential diagnoses vary among patients with different presentations. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted; ‡ Although patients with a score of < 6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time; § Joint involvement refers to any *swollen* or *tender* joint on examination, which may be confirmed by imaging evidence of synovitis; # "Large joints" refers to shoulders, elbows, hips, knees, and ankles; ## "Small joints" refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists; †† Negative refers to international units (IU) values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤ 3 times the ULN for the laboratory and assay; high-positive refers to IU values that are > 3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF; ‡‡ Normal/abnormal is determined by local laboratory standards; §§ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status. ACPA - anti-citrullinated protein antibody; CRP - C-reactive protein; ESR - erythrocyte sedimentation rate.

Evaluation of disease activity must be performed periodically. The disease activity score 28 (DAS28) is a frequent outcome measure used in clinical trials and is also used in therapeutic decisions.

DAS28 is based on count of tender and swollen joints (twenty-eight tender and swollen joints scores including: shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and knees), inflammatory activity (erythrocyte sedimentation rate, ESR and C-reactive protein, CRP) and the subjective evaluation of the global impact of the disease as measured by a visual analogue scale by the patient [315, 316]. By comparing a patient's DAS28 score over time it is possible to substantiate his/her improvement or response to therapy [316, 317].

RA is a complex multifactorial disease with unknown ethiopathogenesis. The process involves environmental triggers and genetic predisposition. Studies in twins suggest that genetic factors account for 60% of the RA onset risk [301, 318]. The HLA system and the protein tyrosine phosphatase non-receptor type 22 (PTPN22) are strongly associated with RA [319-321]. MHC class II HLA-DRB1*0404 and DRB1*0401 alleles have been reported to be strongly associated with this disease [322]. Specifically in the Portuguese population it was also found an association with the DRB1*1001 allele [323]. As T cell specificity is mediated by an interaction between the TCR and the peptide presented by MHC molecules, it has been suggested that RA is caused by the presentation of an unidentified arthritogenic antigen, either exogenous or endogenous [324-326].

The signs and symptoms of RA result from synovitis, the inflammation of the synovial membrane within joints. A complex network of cells and cytokines are involved in the pathogenesis of RA, particularly in the recruitment, activation and effector functions of immune cells. The synovial layers become vascularized and infiltrated with macrophages and fibroblasts in addition to an influx of B and T lymphocytes, plasma cells, mast cells, DCs and neutrophils [302, 327]. The hypertrophic synovial layer, called pannus, contributes to the destruction of bone and cartilage within the joint. IL-1β was the first cytokine reported in the synovial fluid of patients with RA and was linked to cartilage degradation *in vitro* [328, 329]. Subsequently, TNF was also correlated with cartilage damage *in vitro*. It was by this time that TNF was shown to upregulate several other cytokines including IL-1β, IL-6, IL-8 and GM-CSF [330] and of adhesion molecules, such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) [331, 332]. Structural erosions in RA occur along the interface of pannus and cartilage in an area densely populated with TNF producing cells [333]. Synovial fibroblasts, together

with monocyte lineage cells, also promote bone erosion by contributing to osteoclastogenesis [334, 335]. The inflamed synovium invades adjacent cartilage and bone, promoting joint destruction, which is mediated by the activity of OCs, chondrocytes and synovial fibroblasts. In turn, joint damage is a source of antigens, thereby promoting further autoimmune reactivity [336, 337].

In rheumatoid joints, it is well known that the imbalance between pro- and anti-inflammatory cytokine activities contributes to autoimmunity, chronic inflammation and therefore joint damage [253, 338-340]. Bone marrow serves as a site for priming naïve T lymphocytes, recruiting the effector T lymphocytes and increasing their proliferation. T lymphocytes and other immune system cells produce pro-inflammatory cytokines such as TNF, IL-1β, IL-6 and IL-17A that enhance osteoclastogenesis and activate OCs [258]. Cytokines, such as TNF and IL-17, released from activated T lymphocytes, can target bone lining cells and fibroblasts and increase RANKL production, promoting OC activity [253, 341]. Synovial T lymphocytes from RA patients produce low amounts of IFNγ but secrete TNF that induces RANKL expression in synovial fibroblasts. Moreover RANKL is also expressed by activated T lymphocytes, of which Th17 are a major source [258, 342, 343]. Effector T cells (Teff) action is under the tight control of regulatory T cells (Treg) and these cells have been shown to directly inhibit osteoclastogenesis [269, 270]. Despite large numbers of Tregs present in the inflamed synovium of RA patients, their ability to suppress cytokine production or prevent cartilage and bone erosion is impaired due to down regulation of forkhead box P3 (Foxp3) expression by TNF [268]. The increase in TNF and IL-17 secretion associated with low levels of IFNγ and the absence of IL-4 in the synovial fluid may account for the lack of regulation of OC activity [216, 344].

The development of osteoporosis secondary to inflammation in this disease has been demonstrated by increased risk of vertebral and hip fractures, independently of corticosteroid use [345, 346]. Experimental evidences support the view that the peripheral monocyte population changes during inflammation towards an increase in the number of OC precursors [347] and that inflammatory $CD14^{+}CD16^{+}$ monocytes (both intermediate and non-classical subsets) accumulate in the joint, promoting expansion of Th17 cells and turning the balance in favour of OCs [348, 349]. OCs and the RANK/RANKL/OPG pathway are the major players in RA associated bone loss [350-352]. On the other hand, TNF produced by inflammatory cells induces the up regulation of sFRP and DKK proteins inhibiting the Wnt signalling pathway and compromising OB function and matrix

mineralization [275, 353]. Thus, OB differentiation and function are also abnormal in this disease, leading to the uncoupling of bone formation and bone resorption.

RA treatment

All RA patients are treated with disease-modifying anti-rheumatic drugs (DMARDs) like hydroxychloroquine, chloroquine, sulfasalazine, methotrexate (MTX) and leflunomide [354, 355]. If the conventional DMARDs fail, biotechnological drugs ('biologics') are introduced [356]. Patients are also treated with concomitant nonsteroidal anti-inflammatory drugs (NSAIDs) and low dose corticosteroids. NSAIDs reduce joint swelling and pain, however there is limited evidence that these drugs alter the disease course [357]. Low dose corticosteroids are potent suppressors of inflammation. However in high doses glucocorticoids are deleterious for bone as they increase the lifespan of OCs and induce osteoporosis [358, 359]. Previous studies have confirmed that low dose corticosteroids contribute to decrease the progression of RA joint damage [358, 360]. Low dose corticotherapy might be particularly useful in the early phase of the disease and as an add-on therapy on a background of biologic or non-biologic DMARDs that are failing to respond to these drugs [360, 361].

Among the different DMARDs, MTX is the most frequently used and the most likely to induce a long-term response [362-364]. DMARDs have a relatively slow onset of action (1 up to 6 months) but they are effective on the long term and are considered safe [354, 362, 365, 366]. MTX has been shown to reduce the expression of RANKL in synovial fibroblast cultures, which may indicate a specific effect on the OC [367] and more recently was associated with changes in RANKL/OPG ratio and reduction of DKK1 levels [368]. Combination of DMARDs have also proven efficacy [369, 370]. However, around 30% of the patients are either non-responsive to DMARDs therapy or develop adverse effects that are incompatible with their use [365, 371, 372].

Biologics, also referred to as biologic DMARDs, are a class of drugs that has been used and studied for almost 20 years. The first and most frequently used biologics are the group of TNF inhibitors (TNFi): Infliximab, a chimeric monoclonal antibody that neutralizes TNF, and etanercept, the soluble TNF receptor fused to an immunoglobulin region were the first anti-TNF available, followed by adalimumab, a completely human monoclonal antibody targeting TNF [356]. There are currently five TNF inhibitors approved in Europe and in the USA for RA treatment. Of these, three are full-length monoclonal antibodies (infliximab, adalimumab and golimumab), one is a humanized fragment antigen-binding

(Fab) conjugated to polyethylene glycol (certolizumab) and one is a soluble fusion protein (etanercept) [327, 373-376]. TNFi bind with high affinity to soluble and membrane-bound TNF and inhibit its effect by blocking TNF-receptor interactions both in the circulation and in the synovium [375, 377-382]. All TNFi have similar efficacy and the combination therapy with a TNFi plus MTX has been proven to be superior to TNFi alone [365, 383, 384]. When patients fail to respond to one TNFi they have a probability of 50% of responding to a second one [385-387].

In RA, TNFi have shown efficacy in improving clinical, functional and radiographic outcomes [388], especially if administrated early in the disease course [369]. TNF blocking therapies have been shown to arrest radiographic damage in RA and are considered excellent agents for achieving structural protection of joints [374, 389-391]. Indeed, the activation of the destructive process of RA requires higher levels of pro-inflammatory cytokines that even the incomplete inhibition of the signs and symptoms of inflammation will have protective effects on cartilage and bone [392, 393]. TNF is found in high concentration in rheumatoid joints and synovial biopsies and affects the trafficking of cells that can serve as OC precursors, such as $CD14^+$ monocytes. There is also an increase of $CD14^+$ monocytes efflux from the bone marrow and peripheral blood into secondary lymphoid organs and sites of inflammation [334]. The increase in the number of OC precursors in RA patients' PBMCs compared to normal controls appears to be reversible with TNFi therapies and may be a predominant mechanism by which this treatment inhibits erosions in these patients, however there is still no clear understanding of the action of these therapies over the differentiation and activity of OC precursors and OCs [394]. Overall, TNFi have been well tolerated by patients, despite the occurrence of infections and injection-site reactions, which are among the most common adverse effects [327]. However, patients receiving TNFi are more susceptible to bacterial infections (including sepsis and pneumonia), mycobacterium (including tuberculosis) and invasive fungal and opportunistic infections [327, 395, 396].

Despite the beneficial effects of TNFi in the disease progression, only around 60% of the patients respond to this therapy [397]. Over the past decade, the repertoire of biological drugs for RA has rapidly expanded with new inhibitors targeting other pathways of the disease, including tocilizumab (humanized anti-IL-6 receptor monoclonal antibody), abatacept (Fc region IgG1 fused to the extracellular domain of cytotoxic T-lymphocyte-associated protein 4 - CTLA-4) and rituximab (anti-CD20 antibody) [371, 398]. In contrast to the success of TNFi in RA, inhibitors of IL-1β (both IL-1β receptor antagonist anakinra

and the anti-IL-1β monoclonal antibody canakinumab) have been regarded as inferior to TNF inhibitors in suppressing joint inflammation [399, 400]. Treatment of RA patients with tocilizumab is highly effective, with reduced systemic inflammation, synovitis and radiographic progression [401, 402]. Abatacept blocks the interaction of APCs and T lymphocytes at the level of co-stimulatory signalling and shows good clinical efficacy in RA [403]. This CTLA4-immunoglobulin molecule binds to CD80 and CD86 receptors on the APC, selectively blocking the binding to CD28 on T lymphocytes preventing immune responses [403, 404]. Rituximab depletes CD20-positive B lymphocytes by complement-mediated toxicity or apoptosis induction [405-407]. Curiously adverse effects occur with a similar frequency among the different biologics [408]. Moreover, the formation of antibodies to biologic agents is a great concern since it can decrease the efficacy and increase the adverse effects of the treatment [327]. Despite the success of these biological DMARDs, up to 30% of RA patients still may not respond adequately. Many new targets are being tested in early stage clinical trials and new inhibitors of the JAK-STAT signalling pathway are expected to be available soon [356].

Ankylosing spondylitis

The term 'spondyloarthritis' (SpA) designates a diverse group of inter-related types of arthritis that share multiple genetic and clinical features such as inflammatory back pain, asymmetrical peripheral oligoarthritis, enthesitis and specific organ involvement, such as anterior uveitis, psoriasis or chronic inflammatory bowel disease. SpAs are also characterized by the presence of the HLA-B27 antigen [409-411]. Depending on the predominant clinical manifestations SpA can be classified either as axial SpA (characterized by predominant involvement of the spine and/or sacroiliac joints) or peripheral SpA (peripheral arthritis, enthesitis and/or dactylitis) [412, 413]. Axial SpA is characterized by chronic inflammatory back pain and based on clinical and radiological features can be separated in two groups: ankylosing spondylitis (AS) which is defined by the presence of radiographical changes in the sacroiliac joints; and non-radiographic axial SpA which is defined by the presence of sacroiliac inflammation as detected by MRI or the presence of HLA-B27 in combination with the presence of typical features of spondyloarthritis [409, 414]. MHC class I molecule HLA-B27 is the strongest known genetic factor for AS [415]. This antigen is critical for the presentation of antigen-derived peptides to cytotoxic CD8 T lymphocytes [416]. 90-95% of AS patients are positive for

HLA-B27, however only about 10% of HLA-B27-positive subjects are at risk of developing the disease, suggesting that other genes might be involved in disease susceptibility [411, 417, 418].

AS has a prevalence of 0.1-1.4% in the world population [409, 419]. It affects mainly young people at around 26 years of age and is more common in men than women, at a 2:1 ratio [409, 420]. In Portugal, the prevalence of SpA is around 1.6% and AS prevalence has been estimated to be 0.6% [303, 304]. A patient is classified for research purposes to have AS if his/her clinical manifestations match the 1984 Modified New York criteria, which includes both radiological and clinical criteria (see table 2) [409, 421].

Two tools are commonly used to assess disease activity in AS patients: the Bath ankylosing spondylitis disease activity index (BASDAI [422]) and the ankylosing spondylitis disease activity score (ASDAS [423]). BASDAI is a questionnaire based on the patients' perception of fatigue and pain. This index has limitations since it is fully patient oriented, while ASDAS combines patient-reported assessments (like back pain, duration of morning stiffness, patient global assessment of disease activity, peripheral pain or swelling) and also acute phase reactants such as CRP or ESR [424]. The Bath ankylosing spondylitis functional index (BASFI) is used to evaluate the degree of functional limitation and is a questionnaire based on the patients' ability to perform several activities [425, 426].

Table 2 - The 1984 Modified New York criteria for ankylosing spondylitis

A. Diagnosis

1. Clinical criteria
   a) Low back pain and stiffness for more than 3 months which improves with exercise, but is not relieved by rest.
   b) Limitation of motion of the lumbar spine in both the sagittal and the frontal planes.
   c) Limitation of chest expansion relative to normal values corrected for age and sex.
2. Radiologic criterion
   Sacroiliitis grade ≥ 2 bilaterally or sacroiliitis grade 3-4 unilaterally.

B. Grading

1. Definite ankylosing spondylitis if the radiologic criterion is associated with at least 1 clinical criterion.
2. Probable ankylosing spondylitis if:
   a) Three clinical criteria are present.
   b) The radiologic criterion is present without any signs of symptoms satisfying the clinical criteria - other causes of sacroiliitis should be considered.

Apart from chronic inflammation, AS outcome is defined by new cartilage and bone formation [427]. Osteoproliferation can lead to the formation of bone spurs known as syndesmophytes. These are commonly seen in the ligaments of the intervertebral joints, which may result in the progressive ankylosis of the sacroiliac joints and in the fusion of the vertebrae, leading to the appearance of a bamboo like spine, a radiographic feature typical of this disease [416]. Biologically, it is currently believed that AS comprises three phases: i) axial inflammation driven by TNF and other cytokines [428], ii) erosion driven by cathepsin K or MMPs and iii) abnormal bone remodelling driven by BMPs and Wnt proteins [429].

A major question in the molecular pathophysiology of AS is how inflammation and structural damage interact. Although AS is a disease characterized by focal excessive bone formation, it is also a systemic inflammatory disease associated with skeletal bone loss. In fact, low bone mass is a common complication of AS, even in the early stages of the disease [430, 431]. The formation of syndesmophytes occurs at the same location as resolved inflammatory lesions, however there is scarce evidence of correlation between inflammation and new bone formation in AS [391]. Recently, it was shown preliminary evidence of this link by the observation that MRI vertebral corner inflammation followed by fat deposition is the strongest contributor to the development of syndesmophytes at the same location [432].

Imaging and histological studies show that bone destruction is a prominent feature of both axial and peripheral SpA [433, 434]. Accordingly, cellular and molecular pathways of cartilage and bone destruction are activated at the sites of pathology in patients and are largely dependent on TNF [435-438]. On the other hand, it was suggested that the structural features of SpA relate to important pathways of endochondral bone formation. In this process, a cartilage scaffold is formed and BMPs and Wnt signalling play an important role in animal models [439, 440]. Several inhibitors of the Wnt pathway seem to be dysfunctional in AS patients and are associated with new bone formation [441, 442]. There is some debate regarding the need of initial bone erosion in this process but there is a clear role for mechanical stress in the enthesis associated with inflammation and bone formation, at least in animal models of SpA [443-445]. Bone formation in AS is thought to occur by endochondral ossification and to some extent initiated by fatty deposits and direct calcification of the fibrous tissues in both human and animal models [439, 446-450], however the excess bone formation in AS associated with inflammation is not compensated by equal bone resorption also suggesting a dissociation of both processes.

Inflammation in this disease is present both systemically and locally as levels of IL-6 have been shown to be increased in the sacroiliac joints of AS patients [449].

Systemic inflammatory mediators, such as TNF, IL-1β, IL-6 and IL-17, play a role in modulating bone turnover in AS. Serum levels of these cytokines are increased in patients with AS and may be implicated in the development of secondary osteoporosis, since they are known to induce OC differentiation [451, 452]. It has also been shown that OC activity is increased in the bone of AS patients during acute stages of inflammation [453]. TNF has also been identified in synovial and enthesial tissues of AS patients, and TGF-β is present near sites of new bone formation in these samples [454]. In conjunction with IL-6, TGF-β promotes the differentiation of Th17 cells from T cells, thus increasing IL-17 and IL-22 levels. The role of IL-6, TNF and IL-17 has been shown to be pro-osteoclastogenic and anti-osteoblastogenic [455-459] suggesting that in AS there is local deregulated response of OC and OB to these cytokines.

The presence of immune system cells and expression of pro-inflammatory cytokines at the enthesis of AS patients was previously shown [434, 449, 450, 460, 461]. Due to the limited accessibility of the axial skeleton samples, the mechanisms that lead to joint ankylosis and syndesmophyte development in AS are poorly defined. The analysis of zygapophyseal and sacroiliac joints has shown that there is synovial proliferation, fibrosis of synovial tissue, and that cartilage destruction is common. It was also found that radiographic joint space narrowing correlated with cartilage degeneration while the presence of radiographic erosions correlated with the presence of fibrous tissue within the marrow space [462-466]. Interestingly, studies on both zygapophyseal and sacroiliac joints showed that AS patients had increased number of OC and less OB than healthy donors [449, 461, 464, 465, 467]. These studies, however, are limited to sample availability, constraints on disease duration and lack of appropriate controls.

It was also proposed that osteoproliferation in AS is, at least partly, uncoupled from inflammation. Two major hypotheses support this suggestion. The first claims that osteoproliferation can be explained by the intermittent nature of the inflammation [468]. In an early disease phase, TNF would simultaneously drive destruction and up regulate DKK1, thus inhibiting remodelling mediated by the Wnt pathway. By down-regulation of TNF in a later phase, the brake on Wnt mediated remodelling would be released and the early erosions would trigger reactive osteoproliferation. The relation between early inflammation and subsequent new bone formation is, however, still highly debatable in human ankylosing spondylitis because although inflammation is associated with an

increased likelihood of new bone formation, many syndesmophytes are located at sites where no inflammation is detected [469, 470]. Moreover, this hypothesis does not explain why new bone formation is independent of bone resorption in animal models [471, 472].
The second hypothesis, based in animal studies, proposes that direct activation of stromal pathways, including BMPs, leads to new tissue formation independently of inflammation or early erosive changes [471]. Mechanical stress at the synovio-entheseal complexes might then induce distinct and unrelated pathways of inflammation and tissue remodelling [473].

AS treatment

NSAIDs are highly effective in reducing symptoms as inflammation and acute and chronic pain in AS and are thus the first-line therapy for AS patients [474, 475]. Studies suggest that continuous dosing with NSAIDs rather than the usual on-demand prescription decelerates radiographic progression of axial disease over 2 years [476-478]; other trials have however cast doubts on this original observation [479, 480]. However, about half of patients report insufficient control of their symptoms using NSAIDs alone [481-483].
The use of DMARDs for the treatment of axial disease in AS has been rather disappointing [475]. Treatments that are effective in suppressing disease activity and slowing progression in RA have notably failed in SpA, especially in patients with axial disease [482, 484]. Studies reviewed by Dougados *et al.* in 2011 have shown that treatment with DMARDs has no proven efficacy for either the axial or enthesitis features of SpA and only some efficacy for peripheral arthritis and other extra articular manifestations such as psoriasis, uveitis, and inflammatory bowel disease [485-490].
Biologic therapies such as TNFi have been a great advance in the treatment of AS and have become the standard of care for patients refractory to NSAIDs [491-495]. Disease activity and spinal inflammation detected by MRI are substantially reduced by TNFi [475, 496-498]. TNFi therapy is able to, at least partially, halt mechanisms driving cartilage and bone destruction [499] but whether it is able to stop AS radiographic progression has not yet been proven. TNF blockade is highly effective in targeting the different disease features, not only axial disease, but also peripheral arthritis, enthesitis and extra-articular features, such as psoriasis, uveitis and general symptoms, such as fatigue. It substantially improves the overall function and quality of life [500-503]. The safety profile of TNFi in SpA is similar to the one observed in RA patients [504]. The various TNFi seem to be equally potent for the treatment of SpA and there is no recommendation at present to combine TNF

blockers with non-biological DMARDs. Despite its efficacy, around 30% of the patients do not respond adequately to treatment and there are no reliable predictors of response [505, 506]. It seems that patients early in the course of the disease, with high CRP, positive MRI findings and low structural damage are more likely to respond than patients with advanced disease [505]. In case of failure of a first TNF blocker, trying a second drug is justified since many patients still respond to a different TNFi [491].

TNF blockade seems to halt joint destruction [507], but fails to substantially slow new bone formation in SpA [508-510]. It remains unclear whether this effect is related to the fact that TNF blockade was started too late in the disease course in these studies or to the fact that new bone formation is uncoupled from TNF-driven inflammation in SpA. Recent studies and meta-analysis have shown that TNF inhibiting therapies in AS patients are associated with increased lumbar and hip BMD [511]. Moreover, patients have decreased CTX-I levels after TNF-blocking therapy, with increased BMD in lumbar spine [512].

Although inflammation can be treated successfully with TNFi, some follow-up studies have shown a lack of effect on syndesmophyte formation, suggesting that additional factors are governing this effect [391, 508-510, 513, 514]. In one observational study, however, patients with AS taking TNF inhibitors were followed for up to 5 years, and TNF inhibition appeared to reduce radiographic progression [515]. Daily or high use of NSAID has been associated with less radiographic progression, but a truly effective treatment against new bone formation in AS has yet to be found [476, 478, 516-518]. Parallel to the osteoproliferation, patients with AS also have an increased loss of trabecular bone, resulting in an elevated risk for vertebral fractures, even in early and mild AS, due to reduced shock absorption and increased vertebral rigidity [519-525]. Prevalence of osteoporosis of 19% to 62% and vertebral fractures of 9% to 42% have been reported from different AS cohorts [431, 523, 526, 527]. Osteoproliferative changes cause an artefactual increase in lumbar BMD and it has been suggested that the use of lateral projection or hip dual-energy X-ray absorptiometry (DXA) should be used [519-521, 523, 525, 527-531]. These two enhanced but opposite bone processes create diagnostic and therapeutic problems.

It was hypothesised that because TNF can stimulate DKK-1 production, blocking TNF pharmacologically might stimulate new bone formation by decreasing this repressor [469]. However, recent studies showed evidence against this hypothesis by finding that TNFi use was associated with a lower likelihood of radiographic progression and with lower number of new syndesmophytes in 8 years of treatment [532, 533]. TNF blockade seems to halt

joint destruction in other spondyloarthritis, like psoriatic arthritis [507], but fails to substantially slow new bone formation [508-510]. Observational studies of this topic are difficult to design and given the slow rate of syndesmophyte development, clinical trials are not practical using radiographic endpoints, and would require more sensitive measures of syndesmophyte growth.

It remains unclear whether this effect is related to the fact that TNF blockade is started too late in the disease course in these studies or to the fact that new bone formation is uncoupled from TNF-driven inflammation at least in models of arthritis [472]. Follow-up studies associated the use of TNFi to new syndesmophyte formation, which occurs when systemic inflammation decreases (normalization of IL-6 and CRP levels) and ceases when it persists [534, 535]. As such, inflammation and osteoproliferation are at least partially uncoupled events in AS, because osteoproliferation occurs even when TNF is blocked [436, 448, 472]. The precise effect of TNFi in the bone formation/resorption balance remains elusive in AS.

Although RA and AS are two chronic immune mediated diseases their effect on bone metabolism is different. While RA is essentially associated with erosive joint bone lesions, AS induces osteoproliferation at specific sites of the skeleton, pointing towards the existence of subtle differences between RA and AS in the regulation of osteoclastogenesis. Due to excessive proliferation without being compensated by resorption, we hypothesize that osteoclasts from AS patients have an impairment either in differentiation or activity.

Aims

The main goal of the present work was to study the differentiation and activity of circulating OC precursors from RA and AS patients with active disease and the effect of conventional DMARDs and TNFi therapy in these same precursors.

The specific aims of this study were:

i. To study the circulating levels of cytokines in early untreated RA patients and before and after exposure to conventional DMARD therapy;

ii. To characterize the differentiation and activity of osteoclast circulating precursors in a cohort of RA patients before and after exposure to conventional DMARD therapy;

iii. To understand the effect of TNFi in the circulating osteoclast precursors and their differentiation and activity in RA patients;

iv. To study the circulating levels of cytokines and the differentiation and activity of osteoclast circulating precursors in untreated AS patients.

v. To analyze the circulating levels of cytokines and the differentiation and activity of osteoclast circulating precursors in a cohort of AS patients before and after exposure to TNFi.

Results

In agreement with the Decreto-Lei 388/70, art. 8º, parágrafo 2, the results presented and discussed in this thesis were published or submitted for publication in the following scientific peer-reviewed journals:

I. Rita Cascão†, Rita A Moura†, **Inês Perpétuo**, Helena Canhão, Elsa Vieira-Sousa, Ana F Mourão, Ana M Rodrigues, Joaquim Polido-Pereira, Mário V Queiroz, Henrique S Rosário, Maria M Souto-Carneiro, Luis Graca†, João E Fonseca†; *Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis.* Arthritis Research & Therapy 2010 12:R196.

† contributed equally to this work.

II. **Inês P. Perpétuo**, Joana Caetano-Lopes, Ana Maria Rodrigues, Raquel Campanilho-Marques, Cristina Ponte, Helena Canhão, Mari Ainola, João E. Fonseca; *Methotrexate and low dose prednisolone downregulate osteoclast function by decreasing receptor activator of nuclear factor κB expression in monocytes from early rheumatoid arthritis patients* (submitted to RMD Open 2016)

III. **Inês P. Perpétuo**, Joana Caetano-Lopes, Ana Maria Rodrigues, Raquel Campanilho-Marques, Cristina Ponte, Helena Canhão, Mari Ainola, João E. Fonseca; *Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Rheumatoid Arthritis* (submitted to BioMed Research International 2016)

IV. **Inês P. Perpétuo**, Joana Caetano-Lopes, Elsa Vieira-Sousa, Raquel Campanilho-Marques, Cristina Ponte, Helena Canhão, Mari Ainola, João E. Fonseca; *Ankylosing spondylitis patients have impaired osteoclast gene expression in circulating osteoclast precursors* (submitted to Frontiers in Medicine)

V. **Inês P. Perpétuo***, Rita Raposeiro*, Joana Caetano-Lopes, Elsa Vieira-Sousa, Raquel Campanilho-Marques, Cristina Ponte, Helena Canhão, Mari Ainola, João E. Fonseca; (2015) *Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Ankylosing Spondylitis.* PLoS ONE 10(12): e0144655.doi:10.1371/journal.pone.0144655

* Inês P. Perpétuo and Rita Raposeiro contributed equally to this work.

I. Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis.

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† contributed equally to this work.

Inês P. Perpétuo carried out the cytokine quantification in both serum and synovial fluid, performed statistical analysis and wrote the manuscript draft in collaboration with Rita Cascão and Rita A. Moura.

Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early Rheumatoid Arthritis

Cascão, Rita; Moura, Rita A.; Perpétuo, Inês; Canhão, Helena; Sousa, Elsa; Mourão, Ana F.; Rodrigues, Ana M.; Polido-Pereira, Joaquim; Viana Queiroz, Mário; Rosário, Henrique S.; Souto-Carneiro, Maria M.; Graca Luis[†]; Fonseca, João E.[†]

Abstract

Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by sustained synovitis. Recently, several studies have proposed neutrophils and Th17 cells as key players in the onset and perpetuation of this disease. The main goal of this work was to determine whether cytokines driving neutrophil and Th17 activation are dysregulated in very early rheumatoid arthritis patients with less than 6 weeks of disease duration and before treatment (VERA). **Methods:** Cytokines related to neutrophil and Th17 activation were quantified in the serum of VERA and established RA patients and compared with other very early arthritis (VEA) and healthy controls. Synovial fluid (SF) from RA and osteoarthritis (OA) patients was also analyzed. **Results:** VERA patients had increased serum levels of cytokines promoting Th17 polarization (IL-1β and IL-6), as well as IL-8 and Th17-derived cytokines (IL-17A and IL-22) known to induce neutrophil-mediated inflammation. In established RA this pattern is more evident within the SF. Early treatment with MTX or corticosteroids led to clinical improvement but without an impact on the cytokine pattern. **Conclusions:** VERA patients already display increased levels of cytokines related with Th17 polarization and neutrophil recruitment and activation, a

dysregulation also found in SF of established RA. Thus, our data suggest that a cytokine-milieu favoring Th17 and neutrophil activity is an early event in RA pathogenesis.

Introduction

Rheumatoid arthritis (RA) is the most common chronic autoimmune disease, affecting approximately 1% of the population worldwide. This disease comprises a syndrome of pain, stiffness and symmetrical synovitis which leads to joint destruction, functional disability and substantial comorbidity due to involvement of multiple organs and systems. The migration of leukocytes towards the synovium is crucial for the establishment of a chronic inflammatory process in RA [1-3]. This multi-regulated mechanism involves interactions with endothelial cells through cell adhesion molecules and complex cytokine and chemokine pathways.

Neutrophils specifically play an important role in the onset and perpetuation of RA, not only as interleukin producing cells, but also for being responsible for the release of high amounts of reactive oxygen species (ROS) and destructive enzymes, such as metalloproteases, contributing to joint erosions [4]. Neutrophils are among the first leukocytes to arrive at sites of inflammation. In fact, these cells are the most abundant in the synovial fluid of patients with active RA and previous results from our group showed that the synovial tissue is heavily infiltrated by neutrophils in the first weeks of RA onset [5]. Interestingly, in animal models of arthritis, neutrophil depletion prevented joint inflammation if neutrophil depleting antibodies were given before the induction of arthritis. Moreover, when the depleting antibody was given very early after the induction of arthritis complete abrogation of the inflammatory symptoms was also achieved [6].

T helper (Th)-17 cells have also been proposed to have a relevant role in the early phase of RA through the production of interleukin (IL)-17 [7, 8]. This cytokine promotes the recruitment and survival of neutrophils, induces the secretion of proinflammatory cytokines, the upregulation of RANK ligand (RANKL) and stimulates the activity of matrix metalloproteases, leading to cartilage catabolism and bone resorption [9, 10]. The recruitment, activation and effector function of Th17 cells and neutrophils are driven by a network of cytokines and chemokines, secreted by multiple cellular sources. In established RA, it has been reported that IL-1β, IL-6, IL-8, IL-17, and tumor necrosis factor (TNF) are elevated in the serum and this correlates with a higher disease activity [11-13]. Nevertheless, our knowledge on the influence of the cytokine network on RA onset remains scarce. The characterization of the cytokine profile at this stage, where the transition from an acute to a chronic inflammatory phase occurs, may lead to the identification of early key players, with potential implications for early treatment strategies.

Thus, the main goal of our work was to determine whether cytokines driving neutrophil and Th17 cell activation and pro-inflammatory function were already present in very early RA (with less than 6 weeks of disease duration) and how this early cytokine environment differs from established RA. We also evaluated whether the introduction of low dose corticosteroids and methotrexate (MTX) therapy had any influence on the cytokine profile observed at that early stage of the disease.

We found that cytokines related with Th17 polarization and neutrophil recruitment and activation were elevated in early RA and that the conventional therapeutic options, although able to control clinical manifestations of the disease, were ineffective in reversing this underlying pro-inflammatory drive.

Material and methods

Patients

Blood samples were obtained from 38 consecutive untreated polyarthritis patients with less than 6 weeks of disease duration. Some of these patients (19) after a minimum follow up of 3 months fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA [14]. These patients were classified as Very Early Rheumatoid Arthritis (VERA) patients and further samples were collected 4-6 weeks after starting a low dose of oral corticosteroids (5-10 mg of prednisone) (Time 1) and 4 months after reaching the minimum effective dose of MTX (Time 2), up to a maximum of 20 mg/week, that was required to reduce the 28 joints disease activity score (DAS 28) to less than 3.2 [15]. The remaining early arthritis patients (19), who did not evolve into RA, were classified as very early polyarthritis (VEA). Baseline blood samples from VERA and VEA patients were compared with 27 healthy donors used as controls. Additionally, 12 blood and 15 SF samples were obtained from established RA patients. SF samples were also collected from 10 OA patients (Rheumatology Department, Hospital de Santa Maria, Lisbon) (Table 1). Due to the clinical characteristics of the very early arthritis patients, SF in easily accessible joints was not available in VERA and VEA patients and thus SF was not analyzed in these groups of patients. The health assessment questionnaire (HAQ) [16] and DAS28 were applied to all patients. The study was approved by the local ethics committee and all patients signed an informed consent. Patient care was conducted in accordance with the standard clinical practice and the study was performed in accordance with the Declaration of Helsinki as amended in Edinburgh (2000).

Cytokine quantification

IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17A, IL-22, IL-23 and gamma interferon (IFN-γ) levels were measured in the serum and SF by FlowCytomix assay kit (FC) (Bender MedSystems, USA) according to the manufacturer instructions. Standard curves for each cytokine were generated by using reference cytokine concentrations supplied by the manufacturer. Samples were acquired with a FACS Calibur (BD). Raw data of the FC bead assay were analyzed by FlowCytomixPro2.2 software.

Measurement of autoantibodies

Rheumatoid factor (RF)-IgM was determined in all patients by IMTEC Autoimmune Diagnostics ELISA kit (Human GmbH, Germany) according to the manufacturer instructions and samples were processed using a ChemWell® 2910 automated analyzer. Serum levels of anti-cyclic citrullinated peptide (anti-CCP) were measured by ELIA™ CCP test system (Phadia GmbH, Germany) and samples were analyzed using an ImmunoCAP® 100 instrument.

Statistical analysis

Statistical differences were determined with non-parametric Kruskal-Wallis, Mann-Whitney and Wilcoxon Signed-Rank tests using GraphPad Prism (GraphPad, San Diego, CA). Correlation analysis was performed using Spearman's test. Differences were considered statistically significant for $p < 0.05$.

Results

Characterization of patients and disease evaluation.

A total of 38 polyarthritis patients with less than 6 weeks of disease duration were evaluated. Nineteen patients fulfilled the 1987 ACR criteria for RA after a minimum follow-up of 3 months and were classified as VERA patients. The mean age of the VERA patients was 59±17 years-old, 84% were female, 42% were RF positive and 32% anti-CCP positive, the initial DAS 28 was of 6.1±1.8 and the initial HAQ was of 1.4±0.8. After treatment with a low dose of prednisone and MTX there was a significant reduction of both DAS28 and HAQ values (Table 1).

Table 1. Clinical information about healthy controls, VERA, VEA, RA and OA patients.

	Controls	VERA (n=19)			VEA	RA	RA SF	OA SF
	(n=24)	Baseline	Time 1	Time 2	(n=19)	(n=12)	(n=15)	(n=10)
Age in years, mean ± SD	40±13		50±17		40±13	63±10	57±10	67±13
Sex, female/male	17/7		16/3		15/4	11/1	11/4	5/5
DAS28, mean ± SD	NA	6.1±1.8	4.1±1.6*	3.1±1.6*	4.5±1.6*	5.2±1.0	4.6±1.4	NA
HAQ, mean ± SD	NA	1.4±0.8	0.8±0.7*	0.8±0.7	0.8±0.6*	1.5±1.0	1.4±0.8	NA
RF-positive, %	ND	42	ND	ND	0	67	ND	ND
Anti-CCP-positive, %	ND	32	ND	ND	0	45	ND	ND

VEA – very early arthritis; VERA - very early rheumatoid arthritis; RA – rheumatoid arthritis; OA – osteoarthritis; SF - synovial fluid; DAS 28 – Disease Activity Score; HAQ – Health Assessment Questionnaire; RF – Rheumatoid Factor; Anti-CCP – anti-cyclic citrullinated peptide; NA – not applicable; ND – not determined. *DAS28 and HAQ values were compared between VERA and VEA patients with reference to VERA baseline values. Differences were considered statistically significant for p values < 0.05.

The group of VEA patients included 19 patients, who evolved into several different diagnosis: spondylarthritis (5 cases), systemic lupus erythematosus (4 cases), crystal induced arthritis (2 cases), Sjögren's syndrome (1 case), paraneoplastic polyarthritis related

to multiple myeloma (1 case), arthritis associated to HIV infection (1 case). Five patients entered spontaneously into remission before 3 months of follow-up, remaining without a specific diagnosis and were thus classified as presenting a self-limited form of polyarthritis. The mean age of the VEA patients was 40±13 years-old, 79% were female, all patients were RF and anti-CCP negative, the initial DAS 28 was of 4.5±1.6 and the initial HAQ was of 0.8±0.6. Both DAS28 and HAQ values were significantly lower than those of VERA patients at baseline (Table 1). These early polyarthritis patients represent a subset of a larger cohort previously described by our group [17].

Furthermore, blood samples were collected from 12 established RA patients, with a mean age of 60±10 years old, 92% were female, 67% were RF positive and 45% anti-CCP positive (Table 1). Additionally, SF samples were also collected from 12 established RA patients, with a mean age of 57±10 years old and 73% were female (Table 1). The established RA group of patients had a DAS28 and a HAQ mean scores similar to VERA baseline values.

IL-8 is increased in VERA patients and locally in the joints of established RA patients.

Given the proposed role of neutrophils in the pathogenesis of RA [18, 19], we quantified the major neutrophil chemoattractant, IL-8, in the serum of VERA patients. At baseline, VERA patients had significantly higher levels of IL-8 when compared to both VEA and healthy controls (Figure 1A). After 2-4 weeks of low-dose corticosteroids and after 4 months of MTX therapy there were no significant changes in the levels of circulating IL-8 (data not shown). Interestingly, VERA patients also had significantly higher circulating levels of IL-8 in comparison with serum from established RA (Figure 1A). Neutrophils accumulate locally in the joints of RA patients [20]. Thus, we quantified the concentration

of IL-8 in the SF of RA patients, and compared to SF from OA patients. We found significant higher levels of IL-8 in the SF of RA patients in comparison to OA SF (Figure 1B).

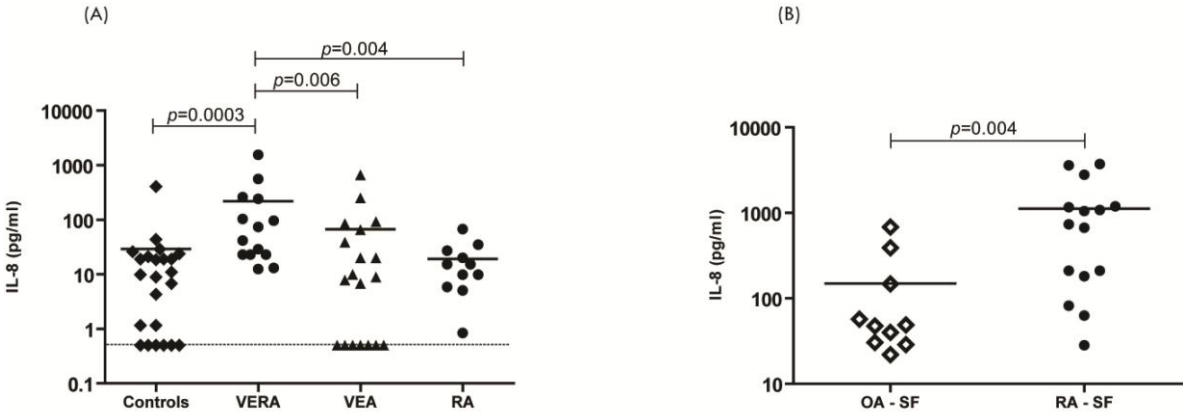

Figure 1. *IL-8 is increased in the serum of VERA patients and in SF of established RA.* **(A)** The serum concentration of IL-8 was measured in VERA and VEA patients as well as healthy controls and patients with established RA. Serum concentration of IL-8 was increased in VERA patients, compared with any other group. Dotted line represents the limit of detection for the assay. **(B)** The concentration of IL-8 was measured in the SF collected from patients with established RA and from a control group with OA. We found a significant increase of IL-8 in RA-SF. Differences were considered statistically significant for p values < 0.05 according to Mann-Whitney test.

IL-17 levels are dysregulated in both VERA and established RA patients.

Previous studies from our group showed that there is a delay in the apoptosis of circulating neutrophils in VERA patients [21]. Therefore, we analyzed IL-17A levels in these patients, since it has already been described that this cytokine is important for the survival of neutrophils [22]. Moreover, IL-17A is a signature cytokine of Th17 cells, a subset proposed to have a key role in RA pathogenesis [9, 23]. We found that VERA patients had significantly higher levels of IL-17A when compared to healthy controls, but not with VEA patients (Figure 2A). Furthermore, in our previous work we found no difference in the

frequency and absolute numbers of $CD4^+$ and $CD8^+$ T cell subpopulations in the peripheral blood of these patients, when analyzed by flow cytometry [17].

Regarding the effects of early therapy, we found that neither corticosteroids nor MTX affected the level of IL-17A (*data not shown*). Moreover, IL-17A was also significantly increased locally within the joints of established RA patients in comparison with control SF from OA patients (Figure 2B).

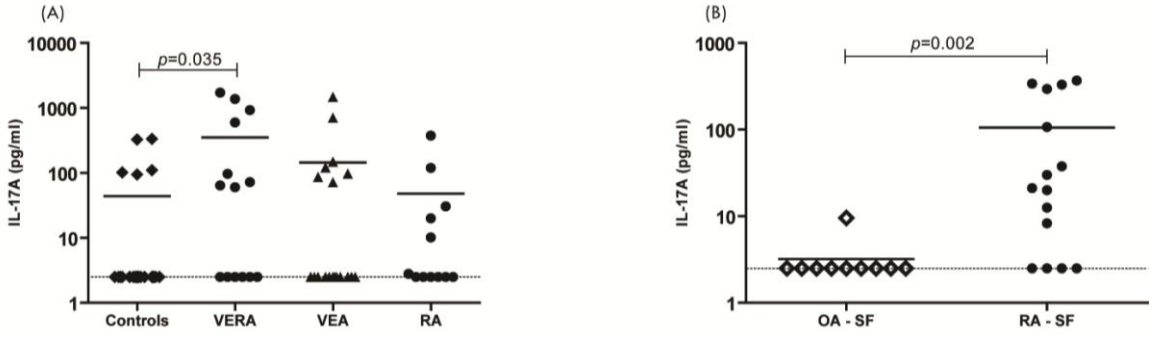

Figure 2. *VERA patients and SF of established RA display increased levels of IL-17A.* **(A)** The serum concentration of IL-17A was measured in VERA and VEA patients as well as healthy controls and patients with established RA. Serum concentration of IL-17A was increased in VERA patients compared with healthy controls. **(B)** The concentration of IL-17A was measured in the SF collected from patients with established RA and from a control group with OA. In the SF of RA patients we observed a significant increase of IL-17A. Dotted lines represent the limit of detection for the assay. Differences were considered statistically significant for p values < 0.05 according to Mann-Whitney test.

RA has a Th17- cytokine pattern since the very first weeks of onset.

Having found that IL-17A was elevated in VERA patients, we decided to quantify a panel of cytokines known to be associated with Th17 polarization. At baseline, VERA patients had significantly higher levels of IL-1β and IL-22 in comparison with both VEA and healthy controls. In addition, we also found that VERA patients have significantly higher IL-6 levels than healthy controls (Figure 3). Furthermore, the significantly higher circulating levels of IL-6 and IL-22 were maintained in established RA (Figure 3).

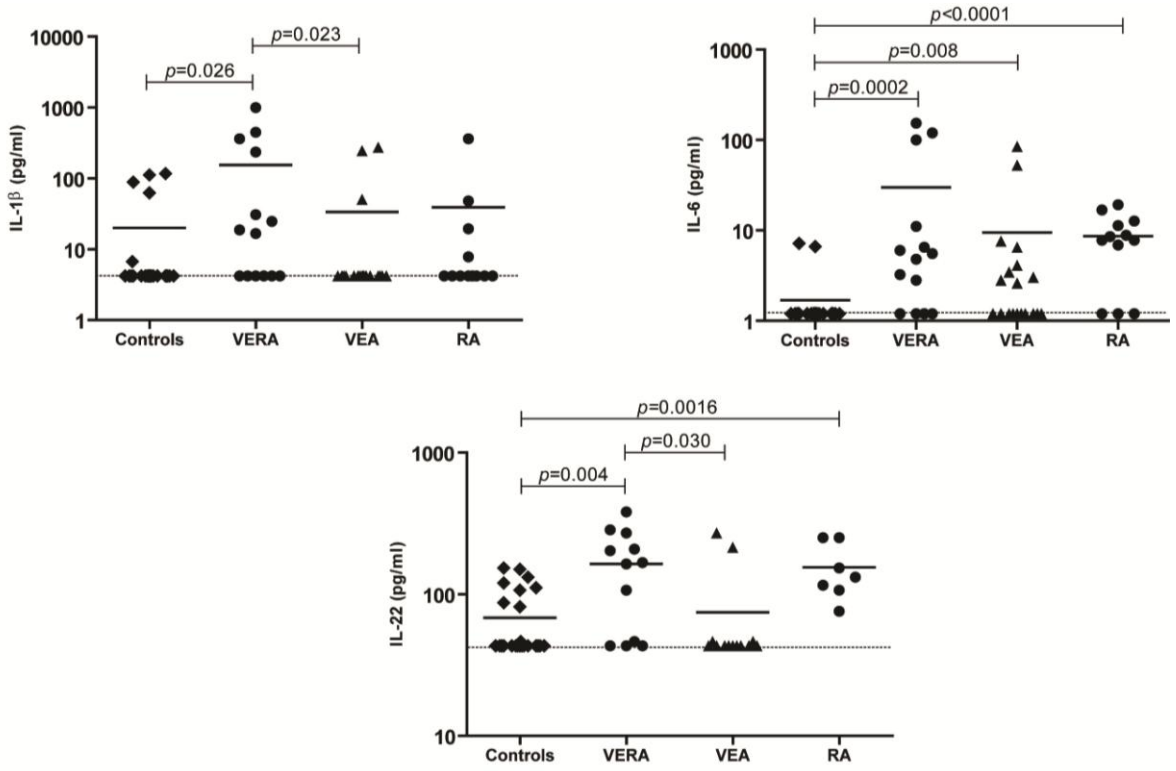

Figure 3. *Cytokines related to Th17 polarization are increased in the serum of VERA patients and SF of established RA.* The serum concentration of IL-1β, IL-6 and IL-22 was measured in VERA and VEA patients as well as healthy controls and patients with established RA. All three cytokines were increased in VERA patients compared with healthy controls. IL-6 was equally elevated in all groups of patients with an inflammatory disease, while the other two cytokines were increased only in VERA (IL-1β), or in VERA and RA patients (IL-22). Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for p values < 0.05 according to Mann-Whitney test.

Locally, within the joints of RA patients, the synovial fluid displayed elevated levels of IL-1β and IL-6 in comparison with OA SF (Figure 4 and Table 2). Moreover, no significant differences could be observed for IL-23 in circulation or locally in the joints (*data not shown*).

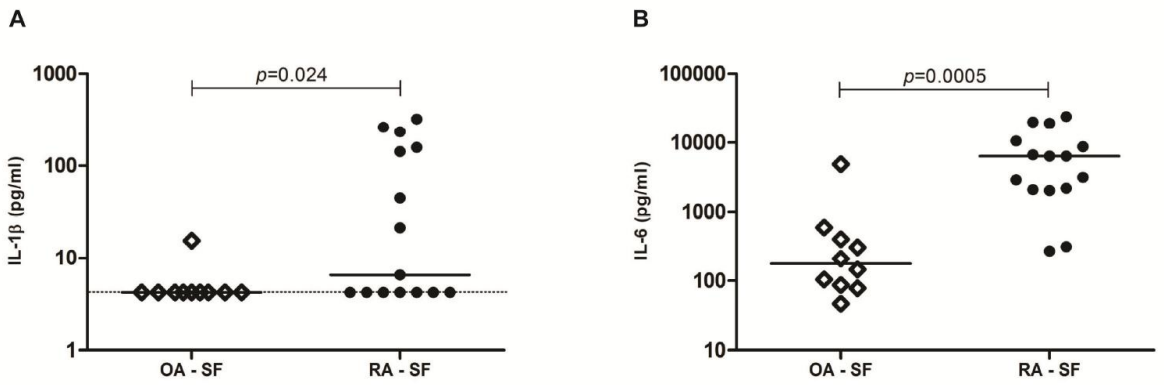

Figure 4. *Cytokines related to Th17 polarization are increased in the SF of established RA* The concentration of IL-1β and IL-6 was markedly increased in the SF collected from patients with established RA, when compared with OA. Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for *p* values < 0.05 according to Mann-Whitney test.

Table 2. Cytokine levels in healthy controls, VERA, VEA, established RA and OA patients.

Cytokine (pg/ml)	Controls	VERA	VEA	RA	RA SF	OA SF
IL-1β	4.2 (4.2-116.8)	17.7 (4.2-99.7)	4.2 (4.2-272.7)	4.2 (4.2-360.3)	6.5 (4.2-322.1)	4.2 (4.2-15.5)
IL-6	1.2 (1.2-7.2)	5.2 (1.2-153.2)	1.2 (1.2-84.7)	8.2 (1.2-19.7)	6361.0 (272.7-24135.0)	177.6 (46.6-4881.0)
IL-8	9.9 (0.5-407.7)	57.6 (12.5-1546.0)	8.9 (0.5-665.2)	15.2 (0.8-67.5)	735.7 (28.3-3717.0)	48.2 (22.0-680.0)
IL-17A	2.5 (2.5-333.7)	62.0 (2.5-1714.0)	2.5 (2.5-1477.0)	2.6 (2.5-375.6)	21.1 (2.5-369.1)	2.5 (2.5-9.5)
IL-22	43.3 (43.3-153.4)	165.3 (43.3-380.6)	43.3 (43.3-270.5)	131.7 (75.8-250.7)	153.4 (75.8-336.0)	151.7 (92.4-235.3)

Values are represented as median (range). VEA – very early arthritis; VERA - very early rheumatoid arthritis; RA – rheumatoid arthritis; OA – osteoarthritis; SF - synovial fluid.

We have also studied cytokines associated with the function of Th2 (IL-4, IL-10) and Th1 (IL-2, IL-12 (p70) and INFγ) cells. However, no statistically significant differences could be observed for any of these cytokines (*data not shown*).

Discussion

Several studies have previously demonstrated that neutrophils play an important role in the onset of RA [21]. This hypothesis is supported by data from animal models [24]. In fact, neutrophils are the most abundant leukocytes in the synovial fluid of patients with active RA and, in early RA, these cells show significantly lower levels of apoptosis when compared to patients with other persistent forms of arthritis or with arthritis that have a self limited disease course [25]. Additionally, previous results from our group demonstrated that there is also a delay in the apoptosis of circulating neutrophils in VERA patients [21] and that these cells heavily infiltrate the synovial tissue during RA onset [5].

In the present study, we demonstrate that a neutrophil- and Th17-driving cytokine pattern is present in untreated VERA patients with less than 6 weeks of disease duration. We consider this observation of interest because the knowledge concerning the immune mechanisms associated with the onset of RA is still elusive. In fact, the majority of early RA studies include patients with 3 to 12 months of disease duration or even more. In accordance with an early participation of neutrophils in RA, our results revealed that VERA patients have increased levels of IL-8 when compared to both VEA and healthy controls, which could explain the pre-activated state of circulating neutrophils [18] and their recruitment towards the synovial fluid since the very first weeks of RA onset.

In addition, Th17 cells are known to be important for the promotion of neutrophil-mediated inflammation by producing IL-17A, a cytokine known to indirectly activate neutrophil chemotaxis and extend their survival [10, 22]. We found a high serum concentration of IL-17A in VERA patients, as well as locally within the joints of patients with established RA. This might indicate that there is an activation of Th17 cells from a very early phase of the

disease, that can promote neutrophil participation in RA pathogenesis [22]. However, we found no evidence for changes in the frequency of T cell subsets in the peripheral blood of VERA patients [17]. This observation is not unexpected, given the relatively small representation of antigen-specific T cells in the circulating pool, being the activated T cells driving the pathology more likely found within the tissues [26]. In a study performed by Kokkonen and colleagues [27] the levels of several cytokines and chemokines were analyzed in blood samples from a group of individuals 3.3 years before RA onset ("pre-patients") and compared with healthy donors and RA patients with 7.7±3.6 months of disease duration. An interesting finding was that IL-17 was present at its highest concentration in pre-patients and the level of this cytokine was lower in RA patients. This is in accordance with our own results, since we observed an increased level of IL-17 in RA patients with less than 6 weeks of disease duration, while in established RA patients the levels were not significantly different to healthy controls. Remarkably, the IL-17 median concentration observed in our established RA cohort (2.6 pg/ml) was even lower than that of RA patients from Kokkonen and colleagues work (6.0 pg/ml). Thus, this observation reinforces the role of IL-17 in the initial phase of RA and, as the pathogenesis progresses to a chronic stage, other factors are subsequently brought into action in the peripheral blood. Contrarily to what was observed in Kokkonen *et al* work we have not detected differences in Th1 and Th2-related cytokines between both VERA and established RA patients in comparison with controls. These discrepancies might be related with the different methodologies used.

Additionally, the elevated levels of IL-1β observed in VERA patients can stimulate endothelial cells, T and B cells and fibroblasts in the joints to produce IL-6 and IL-8. But importantly, IL-1β together with IL-6, both found to be increased in VERA patients, are

known to promote the differentiation of Th17 cells, which in turn secrete IL-17A and IL-22 [28, 29], two cytokines that were elevated in VERA patients and have an essential function in the pathogenesis of autoimmune diseases [29].

Currently, the treatment of choice for RA at the time of presentation is MTX. Interestingly, in spite of clinical improvement (DAS28 reduced from 6.1±1.8 to 3.1±1.6), neither therapy with low-dose corticosteroids nor combined therapy with low-dose corticosteroids and MTX corrected the dysregulated cytokine pattern observed in VERA patients. In fact, low dose corticosteroids and MTX have unclear effects on RA cytokine network. For instance, corticosteroids fail to reduce serum levels of IL-1β and IL-8 [30] and MTX does not alter serum IL-1β concentration when compared to pre-treatment levels [31, 32]. Our results suggest that the conditions contributing to Th17 cells and neutrophil-mediated inflammation, thus driving early pathogenesis, are not modified with early treatment with low-dose corticosteroids and MTX.

The elevated IL-1β, IL-6, IL-8 and IL-17A levels observed in the SF of RA patients confirm a local role for these cytokines in the maintenance of synovitis. Moreover, IL-6 can support a continuous recruitment of autoreactive B cells towards the synovium [33, 34], contributing to an exacerbation of the inflammatory process due to the production of autoantibodies and immune complexes.

Conclusions

Taken together, our data reinforce the potential relevance of therapies targeting IL-1β [35, 36] and IL-6 [37, 38] in early RA. In addition the data also establish IL-8 and IL-17A as other potential therapeutic targets at an early stage of the disease. Finally, we found that

MTX and corticosteroids, although effective in reducing disease activity in VERA patients, do not appear to correct underlying cytokine dysregulation driving the Th17/neutrophil mediated inflammation.

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II. Methotrexate and low dose prednisolone downregulate osteoclast function by decreasing receptor activator of nuclear factor κB expression in monocytes from early rheumatoid arthritis patients

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Methotrexate and low dose prednisolone downregulate osteoclast function by decreasing receptor activator of nuclear factor κB expression in monocytes from early rheumatoid arthritis patients

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Abstract

Objective: Rheumatoid arthritis (RA) is a systemic, immune mediated inflammatory disease that ultimately leads to bone erosions and joint destruction. Methotrexate (MTX) slows bone damage but the mechanism by which it acts is still unknown. In this study we aimed to assess the effect of MTX and low dose prednisolone (PDN) on circulating osteoclast (OC) precursors and OC differentiation in RA patients. **Methods:** RA patients before and at least 6 months after MTX therapy were analyzed and compared with healthy donors. A blood sample was collected in order to assess receptor activator of NF-κB (RANK) ligand (RANKL) surface expression on circulating leukocytes and frequency and phenotype of monocyte subpopulations. Quantification of serum levels of bone turnover markers and cytokines and *in vitro* OC differentiation assays were performed. **Results:** Classical activation markers of monocytes and RANK were increased in RA patients at baseline, comparing to control healthy donors, and after MTX and low dose PDN (MTX+PDN) exposure they decreased to control levels. Although the number of OC was not different between groups, the percentage of resorbed area and the resorbed area/pit were reduced after treatment. Serum RANKL levels were increased at baseline comparing to healthy donors and normalized after

therapy. **Conclusion:** Our results suggest that MTX+PDN play an important role in downregulating OC function, which we believe occurs through the decrease in RANK surface expression in monocytes.

Introduction

Rheumatoid arthritis (RA) is a chronic, immune-mediated, inflammatory disease that mainly impacts joints. It affects around 1% of the world population and the majority of the affected patients are women [1]. The main features of RA are synovial hyperplasia with pannus formation and bone erosions. On the other hand, systemic inflammation leads also to secondary osteoporosis and bone fragility [2]. In RA, there is an unbalance between bone formation and bone resorption. In fact, the immune system contributes directly to bone resorption by producing receptor activator of NF-κB ligand (RANKL) and indirectly through the secretion of pro-inflammatory cytokines that act synergistically with the RANK-RANKL system, further enhancing osteoclast (OC) differentiation and bone resorption [3]. Monocytes are OC precursors; these cells have phenotypical and functional heterogeneity and have a critical regulatory role in inflammation and innate immune response [4, 5]. Monocytes can be subdivided into three sub-populations based on their expression of CD14 and CD16 surface markers: the classical ($CD14^{bright}CD16^{-}$), intermediate ($CD14^{bright}CD16^{+}$) and non-classical ($CD14^{dim}CD16^{+}$) subsets [4]. All of these precursors are able to differentiate into OC increasing bone resorption [6, 7]. Moreover, in RA, $CD14^{+}$ monocytes continuously migrate into the injured joint tissues where they are activated and act as local and systemic amplifiers of the disease through a multitude of mediators [8-10].

Methotrexate (MTX) combined with low dose prednisolone (PDN) is the cornerstone of early RA treatment, reducing inflammation and slowing down bone damage [11-14]. A

recent report depicted that OC formation and OC gene expression, such as NFATc1 and DC-STAMP, which are induced by the cytokine RANKL, are significantly inhibited by MTX [15]. In this study we aimed to assess the effect of MTX and low dose PDN (MTX+PDN) on the circulating OC precursors and OC differentiation in RA patients.

Patients and Methods

Patients

Patients with early untreated RA (less than 12 months of disease duration) fulfilling the American College of Rheumatology/European League Against Rheumatism criteria, 2010 [16] were recruited from the Rheumatology and Bone Metabolic Disease Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. All patients included were naïve to DMARDs and corticoids and were followed in the outpatient clinic during a minimum of 6 months after starting MTX + PDN treatment. Moderate to high disease activity was required for inclusion, defined as disease activity score 28 (DAS28)>3.2 [17]. Patients previously exposed or actively under corticoids and/or DMARD therapy were excluded from this study. The use of non-steroidal anti-inflammatory drugs (NSAIDs) was not an exclusion criterion. Information regarding patients' demographics, duration of symptoms, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), tender and swollen joints counts, presence of erosions, presence of rheumatoid factor (RF) and presence of anti-citrullinated protein antibodies (ACPA) was collected. Both DAS 28 and as the health assessment questionnaire (HAQ) [18] were applied to patients. RA patients were managed by the standard of care approach with no deviation induced by the participation in this study.

Heparinized blood and serum were collected from each participant at the starting of MTX and prednisolone and after 6 months of reaching the minimum effective dose of

MTX. Blood was used for flow cytometry and peripheral blood mononuclear cell (PBMC) isolation. Healthy Donors matched for age and gender were used as controls. The local ethics committee approved this study and all participants signed an informed consent. Patients were managed in accordance with the standard practice and the study was conducted in accordance with the Declaration of Helsinki as amended in Brazil (2013).

Antibodies and flow cytometry

Identification of B and T cells and neutrophils in peripheral blood and immunophenotyping of monocytes in the PBMC samples were performed using matched combinations of anti-human murine mAbs. For peripheral blood staining anti-CD19 PerCP-Cy5.5 (eBioscience, San Diego, CA, USA), anti-CD3 PerCP (BD Biosciences, Franklin Lakes, NJ, USA), anti-CD66b FITC (Immunotools, Friesoythe, Germany) and anti-RANKL PE (Santa Cruz Biotechnology, Dallas, TX, USA) were used. Monocyte subpopulations were identified with anti-CD14 FITC (BD Biosciences) or PerCPCy5.5 (Immunotools) and anti-CD16 APC (Immunotools) and stained with combinations of anti-CD11b PE-Cy7, CD105 PE, CD62L PE-Cy7, CD51/CD61 FITC (eBioscience), CCR2 PE (R&D Systems, Minneapolis, MN, USA), HLA-DR PerCP (BD Biosciences) and RANK PE (Santa Cruz Biotechnology). Cell death was assessed by staining with Annexin V Apoptosis Detection Kit APC (eBioscience). Acquisition was performed usig a FACSCalibur (BD Biosciences).

Heparinized whole blood was used for staining. Erythrocytes were lysed with red blood cell lysis buffer and cells were incubated with IgG block solution 300ng/mL (ChromPure Mouse IgG whole molecule, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) before staining. Absolute cell counts were calculated from differential

leukocyte count determined for all participants. PBMCs were isolated by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). Monocyte subpopulations were identified as described before based on their CD14 and CD16 surface expression [4]. Data was analyzed using FlowJo software (TreeStar, Stanford University, Stanford, CA, USA).

Osteoclast differentiation

PBMCs isolated by density gradient centrifugation were plated in 96-well culture plates as described before [19]. PBMCs were left overnight for osteoclast precursors (OCPs) to adhere on bone slices (Immunodiagnostic Systems, Boldon, UK) [20]. On the following day (day 1 of culture) medium was changed to DMEM supplemented with M-CSF 25 ng/mL (Peprotech, London, UK). Three days later, medium was again changed to DMEM with M-CSF (25 ng/mL), sRANKL (50 ng/mL; Peprotech), dexamethasone (10 nM; Sigma-Aldrich) and TGF-β (2.5 ng/mL; R&D Systems) in order to differentiate the OC precursors into mature OC [21]. The culture medium was then changed twice a week. Cells cultured on bone slices for 7, 14 and 21 days [20, 22] were used for functional assays and gene expression.

Functional assays

TRAP staining of OC was performed using the Acid Phosphate, Leukocyte Kit (TRAP, Sigma-Aldrich) according to the manufacturer's instructions. This assay allows the identification of mature OC (TRAP positive cells containing three or more nuclei). In the resorption assay, to measure the resorbed area, OC were removed from the bone slices using sodium hypochlorite and stained with 0.1% toluidine blue.

Bone slices from both TRAP staining and resorption functional assays were photographed in an area of 1.25 mm2 with a brightfield microscope (Leica DM2500, Leica, Wetzlar, Germany) under a 10x objective. The number of TRAP stained OC was counted for each time-point per condition and the resorption pits were traced using ImageJ (NIH, Bethesda, MD, USA), a mean value of the resorbed area was calculated and expressed in percentage of total area.

Bone turnover markers detection

Serum soluble RANKL and OPG were quantified by ELISA (Biomedica Grouppe, Vienna, Austria) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (IBM, Portsmouth, UK). Categorical variables were expressed as frequencies and comparisons were tested using chi-square test. Continuous variables were expressed by median and interquartile range. Before and after treatment values within each sample were compared using Wilcoxon's matched-pairs signed-rank test. To compare RA patients with healthy age and sex-matched donors Mann-Whitney test was used. *P* values lower than 0.05 were considered significant.

Results

Population characteristics

This study was conducted in 14 untreated early RA patients, before and after initiation of combined MTX and PDN treatment, and in 18 age and gender matched healthy donors. All patients had less than 12 months disease duration and were corticosteroid

and synthetic DMARD naïve at baseline. The clinical and demographic features of patients before and after treatment and of the healthy donors are described in table 1. All patients were under MTX (mean dose of 15 mg/week) and prednisolone (less than 10 mg/day). Hydroxychloroquine (400 mg/day) was added later on 3 patients and 2 patients received concomitant sulfasalazine (2 g/day). Patients' median MTX therapy duration was 13 months (minimum 6 and maximum 24 months).

Table 1 - Summary of patients and healthy controls' characteristics

	RA patients		Healthy	p-value
	Baseline	Treated		
Sample size	14		18	
Age (years)	50 [32-60]		44 [36-52]	0.50853
Females %	71%		61%	0.7120
Symptoms duration (months)	9 [2-12]		NA	
RF(% positive)	57%		NA	
ACPA (% positive)	36%		NA	
Erosive (% yes)	23%		NA	
Treatment with NSAIDs (%)	57%		NA	
NSAIDs duration (months)	2.3 [0.3-5.5]		NA	
Treatment with MTX (%)	0%	100% [a]		
ESR (mm/h)	22 [11-44]	13 [9-27]	NA	0.1934
CRP (mg/dl)	0.3 [0.1-2.7]	0.2 [0.1-0.3]	NA	0.1289
Tender joint count	4 [0-7]	0 [0-1]	NA	0.3738
Swollen joint count	6 [2-10]	1 [0-1]	NA	0.0380*
DAS28	4.7 [4.2-5.4]	2.4 [2.1-3.6]	NA	0.0137*
HAQ	1.4 [0.6-2.2]	0.3 [0.0-0.7]	NA	0.1250

Data is represented as median and interquartile range unless stated otherwise; [a]All patients were under MTX and prednisolone. To 3 patients hydroxychloroquine was added latter on and 2 patients received concomitant sulfasalazine; *$p<0.05$ between patients before and after treatment with MTX; RA – rheumatoid arthritis; NA – not applicable; RF – rheumatoid factor; ACPA - Anti-citrullinated protein antibodies; NSAIDs - non-steroidal anti-inflammatory drugs; MTX - methotrexate; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; DAS – disease activity score; HAQ – health assessment questionnaire.

RA patients had a higher number of neutrophils expressing RANKL than controls, which regressed to control levels after treatment

The number of neutrophils was significantly higher in baseline RA patients when compared to healthy donors (both frequency p=0.0110 and absolute number p=0.0032; Fig.1). Moreover, the absolute number of neutrophils expressing RANKL on their surface was also higher than in controls (p=0.0060). After treatment the absolute counts of neutrophils decreased (p=0.0155). No difference was found in the total number of circulating T or B cells before and after therapy or when compared to healthy donors (data not shown). Moreover, no differences were found regarding RANKL surface expression on neutrophils, T and B cells.

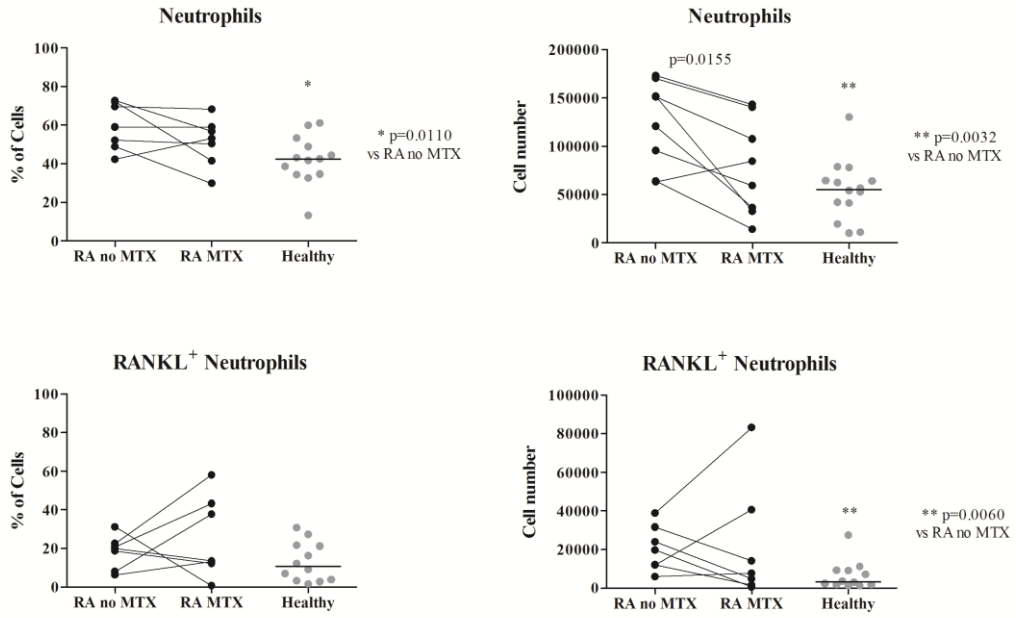

Figure 1. *Frequency of neutrophils and RANKL$^+$ neutrophils.* Neutrophil frequency is significantly higher in RA patients at baseline when compared to healthy donors (both frequency p=0.0110 and absolute number p=0.0032). RANKL$^+$ neutrophil count is also higher at baseline when compared to healthy donors (p=0.0060). Neutrophil number is decreased after MTX and low prednisolone treatment in RA patients (absolute number only, p=0.0155). Each dot represents a sample and the line in healthy represents median. RA - rheumatoid arthritis; MTX - methotrexate; RANKL - receptor activator of nuclear factor κB ligand.

Treatment reduced RANK expression in monocyte subpopulations in RA patients

Regarding the monocyte subpopulations (classical $CD14^{bright}CD16^{-}$, intermediate $CD14^{bright}CD16^{+}$ and non-classical $CD14^{dim}CD16^{+}$) no differences in frequency or cell death among the three subpopulations or between groups were found (data not shown).

In baseline RA patients, CCR2 expression was higher both in the classical and intermediate subpopulations (p=0.0120 and p=0.0471 when compared to controls; Fig.2) and decreased after therapy (p=0.0039 for classical and p=0.0237). HLA-DR and CD86 expression were also higher in the classical subpopulation of RA patients at baseline (p=0.0002 and p=0.0068) as compared to controls. However, while HLA-DR expression remained elevated after treatment (p=0.0006 compared to controls), CD86 expression decreased (p=0.0237). RANK expression was higher than in controls, both in the intermediate and non-classical subpopulations (p=0.0112 and p=0.0475, respectively) while CD51/CD61 ($\alpha_V\beta_3$ integrin) was higher in the intermediate subpopulation (p=0.0487) and CD11b in the non-classical subpopulation (p=0.0160) as compared to controls. Treatment with MTX + PDN decreased RANK and CD11b in the intermediate (p=0.0234 and p=0.0125) and non-classical subpopulations (p=0.0084 and p=0.0132) and CD11b in the classical subpopulation (p=0.0091).

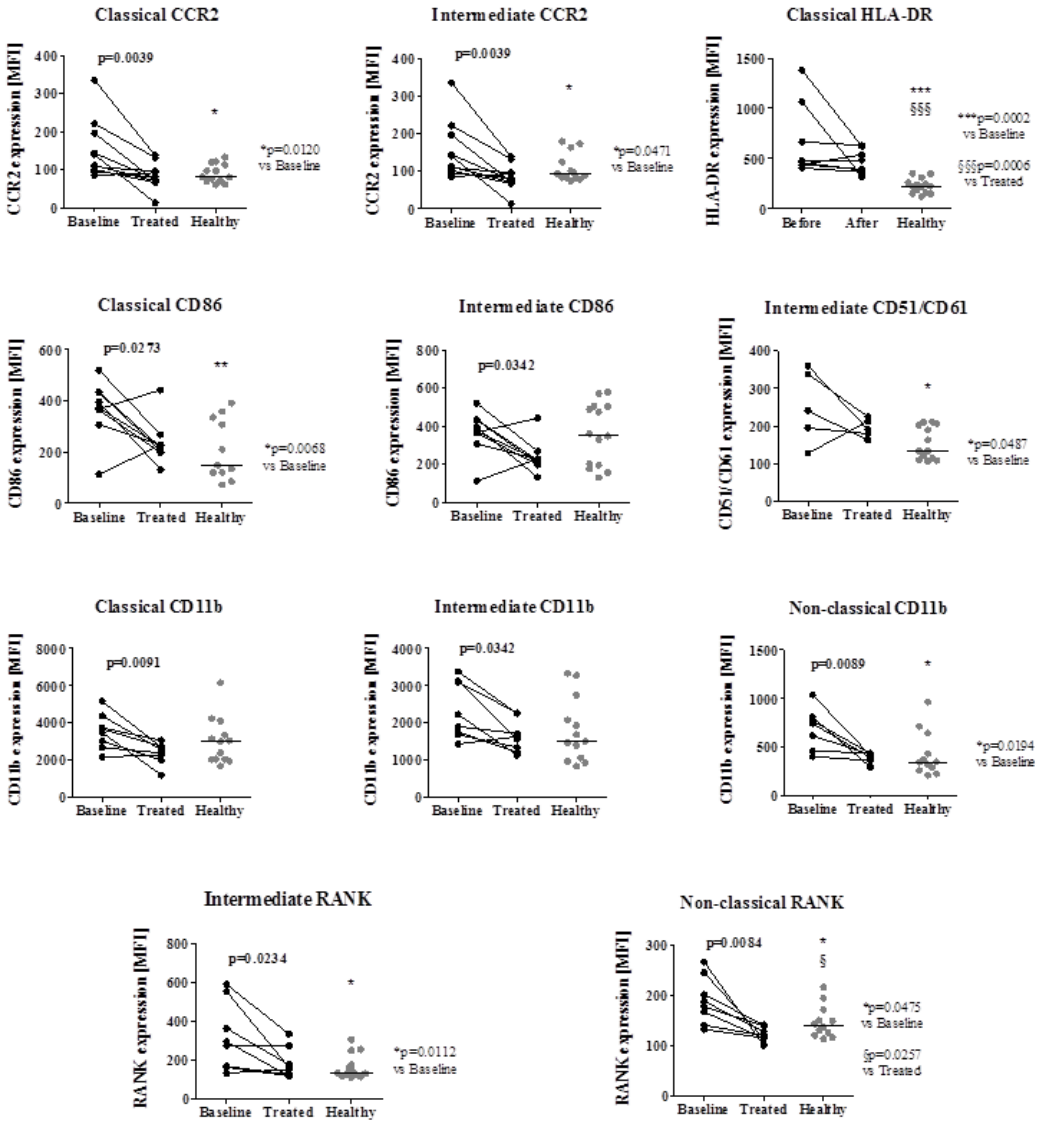

Figure 2. *Phenotype of monocyte subpopulations.* Before treatment there was higher expression of CCR2 in classical and intermediate subpopulations, HLA-DR and CD86 in classical subpopulation, CD51/CD61 in the intermediate subpopulation, CD11b in non-classical subpopulation and RANK both in intermediate and non-classical subpopulations. HLA-DR expression remained higher in patients after treatment when compared to controls. CCR2, CD86, CD11b and RANK were decreased in the referred populations after treatment. Each dot represents a sample and the line in healthy represents median. MFI - median fluorescence intensity; CCR2 - C-C chemokine receptor type 2; HLA - human leukocyte antigen; RANK - receptor activator of nuclear factor κB; MTX - methotrexate.

In vitro assays showed that OCs from RA patients at baseline are more active than the ones from treated patients and controls

The number of OC increased in all groups throughout culture time. No significant differences in OC number/mm^2 or pit number were found (Fig.3). However, the

percentage of resorbed area was higher in baseline patients at culture days 14 and 21 when compared to controls (p=0.0333 and p=0.0436). Resorbed area/pit was higher at culture day 21 before treatment when compared to controls (p=0.0249) and decreased after therapy, reaching statistical significance at day 14 of culture (p<0.0001).

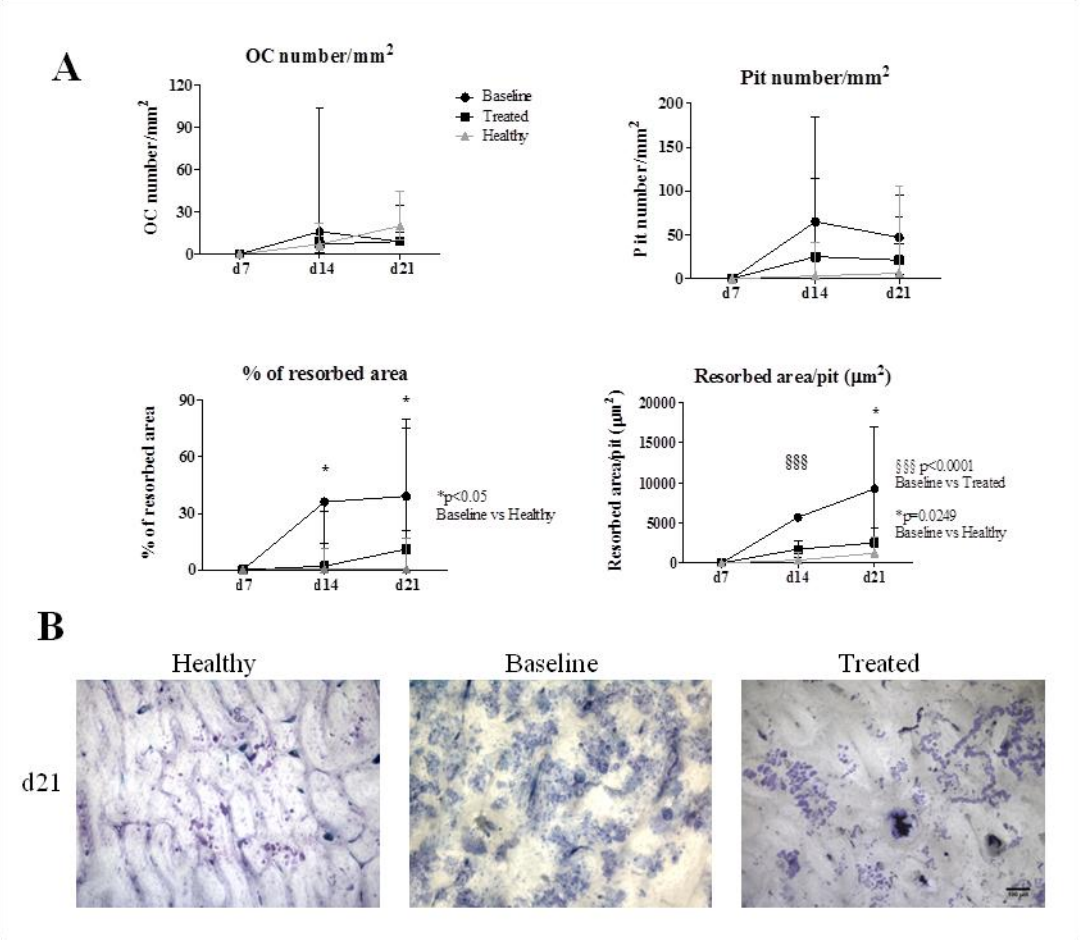

Figure 3. *Functional assays in differentiated OC.* A) No differences in OC number/mm^2 or in pit number were found. Percentage of resorbed area was higher in patients at baseline at culture day 14 and 21 when compared to controls. Resorbed area/pit was lower at culture day 14 when compared to patients at baseline. At culture day 21 RA patients at baseline had higher resorbed area/pit than controls. Dots and lines represent median and interquartile range. B) Representative images of resorption pit assay in all groups at culture day 21; scale bar represents 100µm. d - day; OC - osteoclast; RA - rheumatoid arthritis.

Bone turnover markers were increased in RA patients at baseline and normalized after treatment

Serum level of sRANKL was higher in RA patients at baseline than in controls (p=0.0164; Fig.4) and normalized after treatment. OPG levels did not change with

treatment and was not different from controls. The sRANKL/OPG ratio was higher in patients when compared to controls and it was not affected by treatment (p=0.0228 and p=0.0283 respectively).

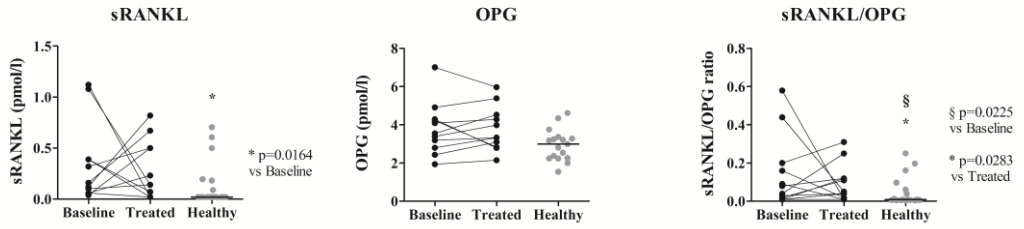

Figure 4. *Serum sRANKL and OPG levels in healthy donors and RA patients at baseline and after MTX and low prednisolone treatment.* Both sRANKL and the sRANKL/OPG ratio were increased in patients at baseline when compared to healthy donors. There was no differences in circulating OPG level before and after treatment or when compared to controls. The sRANKL/OPG ratio remained significantly higher after treatment. Each dot represents a sample and the line in healthy represents median. sRANKL - soluble receptor activator of nuclear factorr κB ligand; OPG - osteoprotegerin; RA – rheumatoid arthritis; MTX - methotrexate.

Discussion

In this study we have shown a decrease in the expression of surface markers on the different monocyte subpopulations, as well as a decrease in OC activity, in early RA patients treated with MTX + PDN.

Neutrophils are involved in RA pathology since the early phase of the disease and they express surface RANKL when activated [23-26]. We showed that the number of neutrophils and $RANKL^+$ neutrophils were increased in untreated RA patients when compared to controls and that, after treatment with MTX + PDN, the number of neutrophils decreased, reaching control values. Despite also being a source of RANKL [27-29] no differences were found in T or B lymphocyte RANKL surface expression before and after therapy.

RA patients have increased expression of monocyte surface markers of adhesion, activation and proteins involved in OC differentiation, like RANK and CD51/CD61 ($\alpha_V\beta_3$ integrin) [30-32]. In our study, after exposure to MTX + PDN, CCR2, CD86, CD11b and RANK expression was reduced in several monocyte subpopulations, reaching normal expression levels. Dendritic cells, also present in circulation, express CD14, CD16 and CD86 [33, 34] and can also differentiate into OC [35, 36]. These cells were also represented in the CD14/CD16 subpopulations of monocytes since we did not exclude them. It was previously shown that CCR2 is up regulated in monocytes and T cells in RA patients. *In vivo* studies have shown that this surface marker is involved in recruitment and activation of monocytes in response to MCP-1 [37, 38]. CD86 is a co-stimulatory molecule involved in the antigen presenting process and it has been shown to have an important role in T cells activation in RA synovia [33]. CD11b expression is increased in RA monocytes, promoting their binding and migrating properties and it has been shown that corticosteroid therapy decreases CD11b expression [39-41]. These surface molecules (CCR2, CD86 and CD11b) are important in migration, activation and promotion of inflammation contributing to the continuous inflammatory infiltrate in the synovia. After therapy with MTX and corticoids these surface markers were reduced in line with a decrease of swollen and tender joints.

We verified that the serum sRANKL/OPG ratio was increased in RA, as previously described [42]. After treatment with MTX and low dose prednisone the sRANKL/OPG ratio decreased. MTX has been recently associated with both reduction of the RANKL/OPG ratio and of dickkopf-1 (DKK-1) levels [43] and it was previously shown that MTX directly inhibits osteoclastogenesis in vitro in synovial fibroblasts and PBMC co-cultures by downregulating RANKL expression on synovial fibroblasts, but no effect on RANK expression was found [44, 45].

Finally, RANK is the essential receptor for monocyte differentiation in OC, it is up-regulated in RA patients and has been implicated in RA bone loss, both systemic and locally [42]. We propose that the decrease in monocyte migration/adhesion molecules and downregulation of RANK expression after therapy decrease the osteoclastogenic potential of these cells. In fact, treated RA patients reached the maximum OC number later than untreated patients and also than controls and the resorbed area per pit decreased significantly after treatment. This suggests that MTX + PDN have an important role in downregulating OC function, which we believe is at least partially through the decrease in RANK surface expression by monocytes.

In conclusion, our results suggest that the EULAR recommended treatment approach for early RA patients (MTX combined with low dose corticosteroids [14]) downregulates OC function, which we propose to occur through the decrease in RANK surface expression by monocytes.

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III. Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Rheumatoid Arthritis

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Inês P. Perpétuo designed the experiments, and performed the flow cytometry, cell isolation, cell culture, functional assays, bone turnover markers quantification, gene expression and image analysis, analyzed the data, produced the figures and wrote the manuscript.

Effect of tumor necrosis factor inhibitor therapy on osteoclasts precursors in rheumatoid arthritis

Inês P. Perpétuo, Joana Caetano-Lopes, Ana Maria Rodrigues, Raquel Campanilho-Marques, Cristina Ponte, Helena Canhão, Mari Ainola, João E. Fonseca.

Abstract

Objective: Tumor necrosis factor (TNF) increases circulating osteoclast (OC) precursor numbers by promoting their proliferation and differentiation. The aim of this study was to assess the effect of TNF inhibitors (TNFi) in the differentiation and activity of OC in rheumatoid arthritis (RA) patients. **Methods:** Seventeen RA patients treated with TNFi were analyzed at baseline and after a minimum follow-up period of 6 months. Blood samples were collected to assess receptor activator of nuclear factor kappa-B ligand (RANKL) surface expression on circulating leukocytes and frequency and phenotype of monocyte subpopulations. Quantification of serum levels of bone turnover markers, *in vitro* OC differentiation assays and qRT-PCR for OC specific genes was performed. **Results:** After TNFi therapy, patients had reduced RANKL surface expression in B lymphocytes and the frequency of circulating classical $CD14^{bright}CD16^{-}$ monocytes was decreased. Serum levels of sRANKL, sRANKL/OPG ratio and CTX-I were reduced in RA patients after TNFi treatment. Moreover, after exposure to TNFi osteoclast differentiation and activity was decreased, as well as the expression of TRAF6 and cathepsin K. **Conclusion:** We propose that TNFi arrests bone loss and erosions, through three pathways: reduction of inflammatory cells numbers, direct reduction of osteoclast precursor numbers, and inhibition of intracellular signaling pathways acting through TRAF6.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by systemic inflammation, bone erosions and secondary osteoporosis [1].

The immune and skeletal systems have several regulatory factors in common and immune system cells have a profound influence on bone metabolism, particularly in the context of chronic inflammatory diseases. Receptor activator of nuclear factor κB ligand (RANKL) is present on osteoblasts' surface, but is also expressed by activated immune cells, both in its membrane form and as a soluble molecule [2]. Tumor necrosis factor (TNF) increases the trafficking of immune system cells that efflux from bone marrow and peripheral blood into secondary lymphatic organs and sites of inflammation and is abundantly found in rheumatoid joints [3]. TNF, together with other cytokines, acts synergistically with the RANK-RANKL system [3, 4], further enhancing osteoclast (OC) differentiation from its circulatory precursors (monocytes) and contributing to bone resorption [2, 5]. It also increases the number of circulating OC precursors and the pro-inflammatory cytokine levels in RA patients. Of interest, TNF inhibitors (TNFi) have a beneficial effect in delaying radiographic damage in RA patients, even in the absence of clinical improvement, suggesting a specific effect of TNF inhibition, independent of inflammation control [6]. Whether this specific effect of TNFi in preventing bone damage in fact occurs independently from the overall inflammatory burden and if it occurs because of reduced OC number and/or function is still unclear.

Our hypothesis was that TNFi decrease OC activity independently of clinical activity response. Thus, the aim of this study was to assess the effect of TNFi in the differentiation and activity of OC precursors in a cohort of RA patients, evaluating also the correlation between clinical and laboratorial manifestations of inflammation and OC related parameters.

Patients and Methods

Patients

Patients with RA fulfilling the 2010 American College of Rheumatology/European League Against Rheumatism criteria [7] were recruited from the Rheumatology Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. All patients included were TNFi naïve and were followed-up during a minimum of 6 months after starting TNFi therapy. Information regarding patients' demographics, duration of symptoms, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), tender and swollen joints counts, presence of erosions, presence of rheumatoid factor (RF) and presence of anti-citrullinated protein antibodies (ACPA) was collected. Disease activity score (DAS28-CRP) was evaluated, as well as the health assessment questionnaire (HAQ) [8].

Heparinized blood and serum were collected at the starting date of TNFi and after a minimum period of 6 months of follow-up. Blood was used for flow cytometry and peripheral blood mononuclear cell (PBMC) isolation. Samples were stored at the Biobanco-IMM, Lisbon Academic Medical Center, Lisbon, Portugal. The local ethics committee approved this study and all participants signed an informed consent. Patients were managed in accordance with the standard practice and the study was conducted in accordance with the Declaration of Helsinki as amended in Brazil (2013).

Flow cytometry

Identification of B and T cells and granulocytes in peripheral blood, RANKL surface expression and immunophenotyping of monocytes in the PBMC samples were performed using matched combinations of anti-human murine mAbs as previously described [9]. Heparinized whole blood was used for staining. Erythrocytes were lysed with red blood cell lysis buffer and cells were incubated with IgG block solution 300ng/mL (ChromPure Mouse

IgG whole molecule, Jackson ImmunoResearch Laboratories) before staining. Absolute cell counts were calculated from differential leukocyte count determined for all participants. PBMCs were isolated by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich). Monocyte subpopulations were identified based on their CD14 and CD16 surface expression [10]. Median fluorescence intensity (MFI) was calculated based only on positive cells as determined by isotype control gating. Data was analyzed using FlowJo software (TreeStar, Stanford University).

Bone turnover markers and bone metabolism proteins detection in the serum

Carboxy-terminal type I collagen crosslinks (CTX-I), human type I procollagen amino-terminal-propeptide (P1NP, Sunred Biological technology), osteoprotegerin (OPG), sclerostin (SOST), dickkopf-related protein (DKK)-1 and soluble RANKL (ampli-sRANKL, Biomedica Grouppe) were quantified by enzyme-linked immunosorbent assay (ELISA) in serum samples according to the manufacturer's instructions.

PBMC isolation and cell culture

PBMCs were isolated by density gradient centrifugation and plated in 96-well culture plates at a density of $7.0x10^5$ cells/well in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5000 U Penicilin/Streptomicin (Invitrogen), 2 mM L-Glutamine (Invitrogen) and 10% Fetal Bovine Serum (FBS; Invitrogen) and incubated in a humidified atmosphere at 37ºC, 5% CO_2. PBMCs were left overnight for OC precursors to adhere on bone slices and were further cultured for 21 days with M-CSF (25 ng/mL, Peprotech), sRANKL (50 ng/mL, Peprotech), dexamethasone (10 nM, Sigma-Aldrich) and TGF-β (2.5 ng/mL, R&D Systems), as described previously [9]. Adherent cells at day 1 and cells cultured on bone slices for 7, 14 and 21 days [11] were used for functional assays and gene expression.

Functional assays

Tartrate-resistant acid phosphatase (TRAP) staining of OCs was performed at days 7, 14 and 21 of culture using the Acid Phosphate Leukocyte Kit (TRAP, Sigma-Aldrich) according to the manufacturer's instructions. OCs were counted as TRAP positive cells containing three or more nuclei [12, 13]. For measurement of resorbed area in the resorption assay at days 7, 14 and 21 of culture, cells were removed from bone slices using sodium hypochlorite and stained with 0.1% toluidine blue [14]. Bone slices from both TRAP staining and resorption functional assays were photographed in an area of 1.25 mm^2 with a brightfield microscope (Leica DM2500, Leica). The number of TRAP stained OCs was assessed at each time-point and resorption pits were traced using ImageJ software (NIH, Bethesda, MD). The resorbed area was calculated and expressed in % of total area.

Gene expression

RNA was extracted from cells cultured over bone slices at days 1, 7, 14 and 21 of culture using NZYol (NZYTech) according to the manufacturer's instructions. Following RNA extraction, total RNA concentration and purity was quantified using Nanodrop 1000 (Thermo Scientific). Complementary (c)DNA was synthesized at a concentration of 0.6 ng/µL using the DyNAmo™ cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's instructions. Genes that encode for osteoclast proteins such as RANK, TRAF6, FRA-2, a subunit of an H^+-dependant ATPase (ATP6V0D2), TRAP and CTSK were studied by real-time quantitative PCR (RT-qPCR). Ribossomal RNA 18s was chosen as the housekeeping gene. Primers (see Suppl. Table 1) were designed using the primer-BLAST software [15] and qPCR was performed using the DyNAmo™ Flash SYBR Green qPCR Kit (Thermo Scientific). The efficiency of qPCR was analysed using the standard curve method [16] as

described previously [17]. The values obtained were normalized with the housekeeping gene 18s rRNA.

Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (IBM). Categorical variables were expressed as frequencies and comparisons were tested using chi-square test. Continuous variables were expressed by median and interquartile range. Spearman's correlations were performed between the analyzed parameters and clinical variables (ESR, CRP, tender and swollen joint count and DAS28). Baseline and post-treatment (follow-up) values within each sample were compared using Wilcoxon's matched-pairs signed-rank test or paired t-test according to distribution normality and p-values lower than 0.05 were considered significant.

Results

Patient background

Seventeen RA patients, evaluated before and after starting TNFi therapy, were included in this study. All patients were receiving methotrexate (weekly 10-20 mg), 15 of them were also under low dose prednisolone and 2 were additionally under bisphosphonates. These therapies had been introduced more than 6 months before TNFi was started and were stable over the study period. Patients were treated with one of four TNFi: one of the monoclonal antibodies (adalimumab, golimumab or infliximab; 41%) or etanercept (59%). A blood sample was obtained before the start of TNFi and after at least 6 months of treatment. Thirteen patients (76%) were good responders to TNFi and 4 (24%) were moderate responders according to the EULAR response criteria [18]. Joint counts, ESR, CRP, DAS28 and HAQ were significantly decreased after TNFi therapy. The clinical and demographic characteristics of patients both at baseline and follow-up are described in Table 1.

Table 1 - Baseline and follow-up characteristics of patients

	RA patients (n=17)		p-value
	Baseline	**Follow-up**	
Age (years)	50 [38-63]		-
% Females	71%		-
Symptoms duration (years)	6 [3.5-9.5]		-
Rheumatoid factor (% positive)	71		-
ACPA (% positive)	53		-
Erosive (% y)	59		-
Treatment with NSAIDs (% y)	47		-
Treatment with DMARD (% y)	100		-
DMARD duration (months)	15 [3-51]		-
ESR (mm/h)	28 [18-48]	21 [13-26]	0.0257
CRP (mg/dl)	1.4 [0.7-2.0]	0.3 [0.04-0.8]	0.0018
Tender joint count	9 [4-14]	0 [0-2]	0.0005
Swollen joint count	7 [4-9]	0 [0-0]	0.0005
DAS28-CRP	5.6 [5.2-6.3]	2.9 [2.2-3.5]	<0.0001
HAQ	1.7 [0.8-2.0]	0.1 [0.0-1.0]	0.0059
TNFi duration (months)	-	6 [6-12]	-

Data is represented as median [Interquartile range] unless stated otherwise; p-value<0.05 is considered significant; ACPA - anti-citrullinated protein antibodies; CRP – C-reactive protein; DAS – disease activity score; DMARDs – disease modifying antirheumatic drugs; ESR – erythrocyte sedimentation rate; HAQ - Health assessment questionnaire; NSAIDs - non-steroidal anti-inflammatory drugs; RA – rheumatoid arthritis; TNFi – tumor necrosis factor inhibitors; y – yes.

TNFi treatment in RA patients decreases the frequency of circulating osteoclast precursors

After TNFi treatment, the frequency of the classical monocyte subpopulation ($CD14^{bright}CD16^{-}$) was decreased (p=0.0065; Table 2) and the non-classical subpopulation ($CD14^{dim}CD16^{+}$ [10]) was increased (p=0.0005). No differences were identified in either CD51/CD61 ($\alpha_v\beta_3$ integrin) or RANK surface expression. After statistical correction for

multiple comparisons only the increase in the non-classical subpopulation remained significant.

Table 2 - Monocyte subpopulation frequency and osteoclastogenic marker expression

	Baseline	Follow-up	p-value
Classic (%) [a]	88 [82 - 89]	78 [70 - 83]	0.0065**
Classic CD51/CD61 MFI	130 [119 - 148]	125 [111 - 137]	0.4258
Classic RANK MFI	133 [116 - 160]	122 [100 - 135]	0.1849
Intermediate (%) [a]	4.4 [2.4 - 5.6]	4.0 [2.1 - 7.1]	0.6013
Intermediate CD51/CD61 MFI	222 [139 - 400]	193 [146 - 240]	0.8203
Intermediate RANK MFI	197 [117 - 361]	188 [120 - 272]	0.9102
Non-classic (%)[a]	5.7 [4.1 - 11]	14 [11.5 - 18.1]	0.0005***,†
Non-classic CD51/CD61 MFI	192 [80 - 290]	142 [127 - 167]	0.5703
Non-classic RANK MFI	139 [122 - 157]	138 [126 - 146]	1.0000

Flow cytometry results are shown as median and interquartile range; [a] gated on the monocyte subpopulation from peripheral blood mononuclear cells. RANK - receptor activator of nuclear factor-κB; MFI - median fluorescence intensity (arbitrary units); **p<0.01, ***p<0.001, † - remained significant after correction for multiple comparisons.

RANKL surface staining was performed in $CD66b^+$ neutrophils, $CD3^+$ T cells and $CD19^+$ B cells (Table 3). No difference was found in the total number of circulating neutrophils and T or B cells after therapy. Although the frequency of $RANKL^+$ neutrophils or T cells was not significantly different after treatment, both frequency and absolute number of $RANKL^+$ B cells were higher post treatment (p=0.0088 and 0.0029, respectively). However, B cell RANKL surface expression was significantly decreased after treatment (p=0.0401). When statistically corrected for multiple comparisons the increase in $RANKL^+$ B cells remained significant.

Table 3 - Whole blood cell distribution and RANKL expression

	Baseline	Follow-up	p-value
Neutrophils (%) [a]	82 [71 - 91]	90 [84 -091]	0.2662
Neutrophils (x10^8cells/L)	12.7 [8.0 - 15.6]	9.6 [8.4 - 12.9]	0.2642
RANKL$^+$ Neutrophils (%) [b]	22 [3 - 41]	53 [21 - 77]	0.0856
RANKL$^+$ Neutrophils (x10^8cells/L)	1.5 [0.3 - 4.3]	5.9 [1.8 - 7.1]	0.1475
Neutrophil RANKL MFI	33.2 [25.5 - 44.9]	24.1 [21.7 - 28]	0.0830
T cells (%) [c]	62 [58 - 74]	68 [52 - 72]	0.5265
T cells (x10^8cells/L)	4.2 [2.4 - 5.2]	3.4 [2.4 - 11.7]	0.4131
RANKL$^+$ T cells (%) [b]	6.2 [0.8 - 24]	6.7 [4.6 - 15.7]	0.8984
RANKL$^+$ T cells (x10^8cells/L)	0.30 [0.03 - 1.03]	0.20 [0.16 - 0.69]	0.7646
T-cell RANKL MFI	49 [41 - 55]	32 [25 - 53]	0.2061
B cells (%) [c]	7.3 [4.8 - 14]	9.2 [4.9 - 15.0]	0.7364
B cells (x10^8cells/L)	0.40 [0.18 - 0.94]	0.44 [0.23 - 1.51]	0.9658
RANKL$^+$ B cells (%)	4.7 [2.0 - 6.7]	14 [3 - 28]	0.0088**
RANKL$^+$ B cells (x10^8cells/L) [b]	0.02 [0.01 - 0.06]	0.06 [0.02 - 1.22]	0.0029**,†
B-cell RANKL MFI	48 [38 - 80]	30 [25 - 63]	0.0401*

Flow cytometry results are shown as median and interquartile range; [a] gated on granulocytes from whole blood; [b] gated on the correspondent parent gate (neutrophil, T or B cell); [c] gated on the non-granulocyte cells from whole blood (also called the "monolymph" gate). RANKL - receptor activator of nf-κβ ligand; MFI - median fluorescence intensity (arbitrary units); *p<0.05, **p<0.01, † - remained significant after correction for multiple comparisons.

The sRANKL/OPG ratio and CTX-I circulating levels are reduced in RA patients after TNFi treatment

Circulating levels of sRANKL were significantly decreased after TNFi (p=0.0085; Table 4), leading to decreased sRANKL/OPG ratio (p=0.0031). We found no differences in the circulating levels of DKK-1 or SOST. CTX-I and P1NP levels were lower in patients at 6 months of follow-up, when compared to patients at baseline (p=0.0005 and 0.0252, respectively) and no difference was found in the CTX-I/P1NP ratio. After correcting for

multiple comparisons, the differences in sRANKL/OPG and CTX-I after treatment remained significant.

Table 4 - Serum levels of bone turnover markers and bone metabolism proteins

	Baseline	Follow-up	p-value
sRANKL (pmol/L)	0.32 [0.21 - 0.67]	0.18 [0.11 - 0.35]	0.0085**
OPG (pmol/L)	4.34 [2.60 - 5.82]	4.22 [3.05 - 5.08]	0.7990
sRANKL/OPG	0.08 [0.04 - 0.17]	0.05 [0.03 - 0.07]	0.0031**,†
DKK1 (pmol/L)	25.5 [18.1 - 43.3]	26.4 [21.9 - 31.7]	1.000
Sclerostin (pmol/L)	25.2 [16.94 - 33.8]	25.2 [19.2 - 29.3]	0.8577
CTX-I (ng/mL)	194.6 [176.6 - 430.7]	163.6 [152.1 - 173.9]	0.0005***,†
P1NP (ng/mL)	55.7 [46.3 - 61.3]	45.8 [39.6 - 48.9]	0.0252*
CTX/P1NP	3.36 [3.09 - 3.82]	3.71 [3.34 - 4.30]	0.5590

Enzyme-linked immunosorbent assay results are shown as median and interquartile range. sRANKL - soluble receptor activator of nf-κβ ligand, OPG - osteoprotegerin, DKK1 – dickkopf related protein 1, CTX - carboxy-terminal telopeptide of type I collagen, P1NP - total procollagen type 1 N-terminal propeptide; *p<0.05, **p<0.01, ***p<0.001, † - remained significant after correction for multiple comparisons.

Osteoclast differentiation and activity in RA patients is decreased after TNFi treatment due to decreased TNF intracellular signaling and cathepsin K expression

Under stimulating conditions, cells from patients treated with TNFi formed less OC than cells from patients at baseline (p=0.0094 at culture day 14, p=0.0203 at culture day 21; Fig.1). Although the number of resorption pits was not significantly different before and after treatment, the area resorbed per pit was significantly reduced in cells from patients at follow-up at culture day 21 (p=0.0038), which resulted in significantly decreased total resorbed area

(p=0.0383). After statistical correction for multiple comparisons only the differences in OC number at day 14 and the resorbed area per pit at day 21 remained significant.

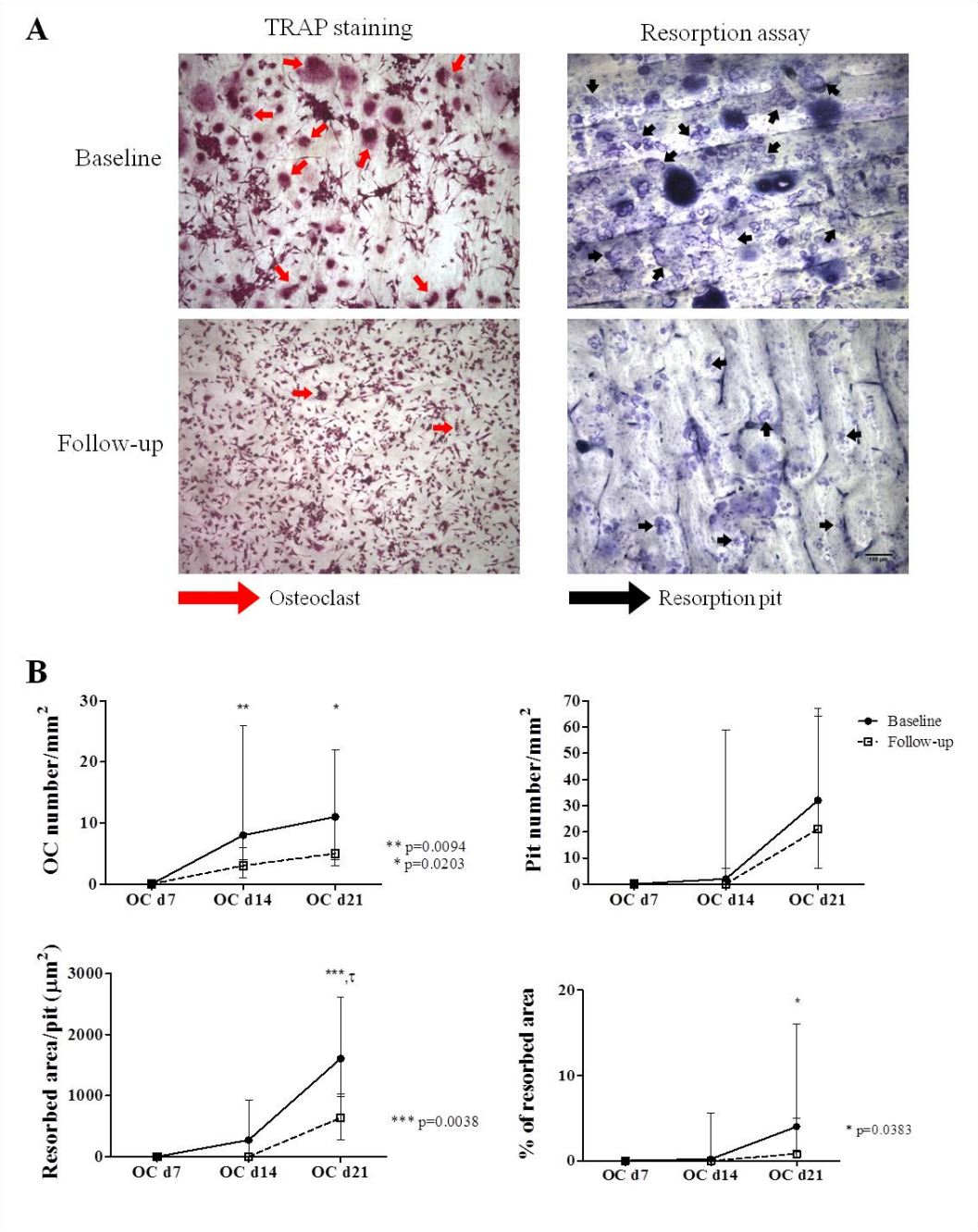

Figure 1. *Osteoclast differentiation from circulating precursors of RA patients before and after TNFi.* A) Representative images, at culture day 21, of cells stimulated with M-CSF, RANKL, dexamethasone and TGF-β stained for TRAP and where the pit assay was performed. B) OC number increased throughout time and at culture days 14 and 21, patients at follow-up have significantly less osteoclasts than at baseline (p=0.0094 and 0.0203, respectively). No differences were found in the number of resorption pits/mm^2; patients at follow-up had significantly smaller pits at culture day 21 (resorbed area/pit, p=0.0038) and significantly less resorbed area at culture day 21, when compared to their baseline (p=0.0383). Dots represent median counts for each group at each time-point and bars represent interquartile range. d - day; OC - osteoclast; Scale bars 100µm, red arrows - osteoclasts, black arrows - resorption pits. τ - remained significant after adjusting for multiple comparisons.

Gene expression by RT-qPCR was performed for OC genes that are known to be important during these cells' differentiation and activity. At culture day 1, TRAF6 expression in patients at follow-up was significantly lower than in patients at baseline (p=0.0229; Fig.2). At culture day 7, expression of both FRA-2 and CTSK was significantly decreased after TNFi treatment (p=0.0242 and 0.0350, respectively). No differences were found in any of the studied genes at culture day 14, but at culture day 21 there was a significant decrease in CTSK expression in cells from patients post treatment. This difference in CTSK expression remained significant after multiple comparisons adjustment.

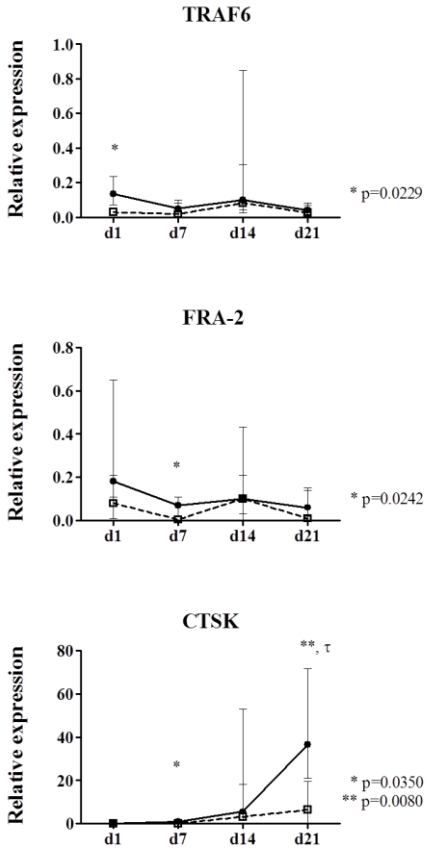

Figure 2. *Gene expression profile of stimulated cells in culture for 21 days.* At day 1 TRAF6 expression in patients at follow-up is significantly reduced (p=0.0229). At day 7, both FRA-2 and CTSK expression is significantly decreased (p=0.0242 and 0.035 respectively). At day 21 patients at follow-up have significantly reduced expression when compared to patients at baseline (p=0.008). Relative gene expression is shown in arbitrary units. Dots in graphs represent median gene expression for each group at each time-point and lines represent interquartile range [25-75]. d - day; TRAF6 - gene encoding for tumor necrosis factor receptor-associated factor 6, FRA-2 - gene encoding for Fos-related antigen 2, CTSK - gene encoding cathepsin K. τ - remained significant after adjusting for multiple comparisons.

No differences were found in any of the studied parameters when comparing monoclonal antibodies (adalimumab, infliximab or golimumab) with the fusion protein etanercept (data not shown). No correlation was found between clinical or laboratorial inflammatory parameters for any of the studied variables.

Discussion

With this study we aimed to test the effect of TNFi in the differentiation and activity of OC precursors in RA patients.

We have shown that RA patients treated with TNFi have reduced frequency of classic monocytes. We also found a decrease in the circulating levels of soluble RANKL and consequently a reduction in the sRANKL/OPG ratio after TNFi treatment. Although no differences in circulating levels of SOST or DKK-1 were detected, serum CTX-I and P1NP levels were decreased after TNFi treatment, reflecting decreased bone turnover in these patients. Accordingly, we found that the *ex vivo* differentiation and resorptive activity of OC precursors from TNFi treated patients was reduced, mainly due to an early downregulation of TNF signaling proteins, such as TRAF6 or FRA-2, and to a later reduction of CTSK expression. Moreover, when comparing all studied parameters we found no differences between the use of monoclonal antibodies (adalimumab, golimumab and infliximab) and the fusion protein (etanercept), suggesting that they have similar effects on OC precursors. Previous studies have compared the effects of different TNFi in disease activity, sRANKL/OPG ratio and circulating leukocytes without finding significant differences [19, 20].

It has been previously reported that granulocyte numbers were reduced in circulation after 2 and 14 weeks of infliximab treatment [21]; however, this study identified granulocytes as $CD16^+$ cells instead of $CD66b^+$ cells. We found no significant differences in the frequency of

neutrophils and T lymphocytes or in RANKL surface expression in these cells, but we observed a significant increase in $RANKL^+$ B lymphocytes accompanied by a decrease in RANKL surface expression. There have been a number of studies addressing the effect of TNFi in RA patients' peripheral lymphocytes, however there is no consensus among different reports, mainly due to sampling differences. In 2005 Toubi and colleagues have shown that infliximab decreased apoptosis in Tregs of RA patients [22]. Other studies showed that short *in vitro* exposure of PBMCs to infliximab or etanercept had no effect in peripheral lymphocyte apoptosis [23] or in synovial membrane biopsies [24]. It has previously been shown that RA patients under TNFi have increased number of T regulatory cells and a reduced number of T effector cells [25]. Other studies showed that in TNFi treated RA patients there were no changes in T regulatory cells frequency [19] or in the frequency of total T cells, monocytes or granulocytes and only a transient unspecified effect on B cells [26]. To our knowledge a comparative study of RANKL expression in RA patients before and after therapy with TNFi has never been published.

Three monocytes subpopulations, based on their expression of CD14 and CD16 surface markers, have been described in humans [10]. In RA patients it has been shown that the intermediate subpopulation is increased when compared to healthy donors [10] and apoptosis of local and peripheral monocytes/macrophages was also increased after etanercept or infliximab treatment [24, 27]. Another study has shown no differences in $CD14^{dim}$ or $CD14^{bright}$ subpopulations after 4 months of infliximab therapy [21]. In our cohort, 6 months after TNFi therapy, patients showed decreased classic ($CD14^{bright}CD16^-$) and increased non-classical subpopulations ($CD14^{dim}CD16^+$). These changes in frequency were accompanied by a non-significant decrease in CD51/CD61 ($\alpha_v\beta_3$ integrin) and RANK surface expression in all subsets. In accordance with our results, a recent study showed a reduction in classical monocyte subpopulation and an increase in the non-classical subpopulation following

infliximab therapy [28]. Moreover, Sprangers et al. observed that although non-classical monocytes can also differentiate into OC, these cells have lower resorptive ability [29], which might explain why we did not observe bone resorption increase.

Patients under TNFi had reduced levels of sRANKL, sRANKL/OPG, CTX-I and P1NP, suggesting a decrease in OC activity and a return to a balanced coupling of bone resorption and bone formation. No differences were found in the circulating levels of DKK1 and SOST after TNFi treatment. Previous studies have shown discrepancies in the determination of these bone remodeling-associated proteins. Studies have found no differences in sRANKL or OPG serum levels after infliximab or etanercept [30, 31]. However contradictory results have emerged regarding both OPG and sRANKL circulating levels after TNFi therapy [32, 33]. DKK1 and sclerostin have a direct effect in bone formation through interaction with the Wnt signaling pathway [34] but they have not been extensively studied in RA patients under TNFi. Previous reports have shown that etanercept has no effect in circulating levels of DKK1 but it increases sclerostin in circulation after treatment [30]. However, infliximab has been shown to decrease DKK1 levels in patients responding to therapy [35]. It has been previously shown that TNFi have a beneficial effect, reducing radiographic damage in RA patients, even in the absence of clinical improvement [6, 36]. Reports have described a decrease in CTX-I or urinary markers of bone resorption after TNFi therapy [30, 37]. However, some discrepancies have been found when studying bone formation markers. Studies with etanercept and infliximab showed no alteration in circulating P1NP levels after treatment [30, 37], while other study with etanercept showed reduced levels of urinary bone formation markers [38].

Although the classical monocyte subpopulation has been considered the OC precursor subset all three subpopulations can differentiate *in vitro* into OC [39]. To understand the effect of TNFi in OC differentiation and function we isolated PBMCs from RA patients before and after TNFi treatment and cultured them *in vitro* over bone slices. After TNFi treatment we

found a decrease in OC number and both in the total resorbed area and in the average resorbed area per pit. No differences in pit number and in the number of nuclei/OC, aspects associated with OC activity, were identified [40]. These observations suggest that TNFi reduces the number and mobility of OC.

Complex *in vivo* studies with animal models also showed that infliximab and etanercept reduced the bone resorbed area [41, 42] and etanercept decreased $\alpha_v\beta_3$ integrin expression [43]. In a study similar to ours, Gengenbacher and colleagues studied RA patients under infliximab therapy for 6 months and observed decreased pit number after *in vitro* cell culture in OC differentiating conditions [31]. There have been reports that infliximab inhibits directly (*in vitro*) murine and human OC formation [44, 45]. Other authors show that although TNFi reduce the number of murine pre-OCs *in vitro*, there is no effect in the total number of formed OC [46]. Another study has shown that infliximab directly inhibits OC formation in high density healthy PBMC cultures without any further stimuli [47]. Etanercept was also shown to inhibited *in vitro* OC formation induced by M-CSF and IL-23 from healthy subjects [48]. Controversially, Takita and colleagues cultured PBMCs from RA patients exposing them to M-CSF, RANKL and infliximab *in vitro*, and observed that infliximab increased bone resorption when compared to M-CSF and RANKL alone [49].

There is evidence that TNF contributes to expression of specific OC proteins and that it directly activates OC differentiation through cross activation of the NF-κB pathway or c-Jun N-terminal kinase (JNK) signaling cascade [50]. We were interested in understanding the underlying mechanisms of reduced OC formation and bone resorption after TNFi so we conducted gene expression assays and observed that OC precursors from RA patients after TNFi exposure had decreased expression of TRAF6 at culture day 1, followed by a reduction of FRA-2 and CTSK at day 7 and finally decreased expression of CTSK at culture day 21, when compared to patients before TNFi exposure. RANK/RANKL signaling cross-talks with

TNF signaling, as RANK is a TNF-superfamily member [51]. Upon activation, both RANK and TNF activate cytoplasmic kinases and adaptor proteins, among them TRAF6, that further activate FRA-2 [52]. Fra-2 is a protein that when associated with Fos and AP-1 promotes the transcription of OC differentiating genes, including CTSK [53]. In TNFi-treated patients we have observed, not only a decrease in serum CTX-I (cleaved by CTSK), but also a reduction in CTSK expression after cell differentiation *in vitro*, as well as a decline in the resorbed area/ OC. This has previously been observed in a RA patient with concomitant pycnodysostosis, an autosomal recessive mutation in the cathepsin K gene characterized by absence of this enzyme. Osteoclasts from these patients form very small resorbing pits and do not release CTX-I into the culture media [54].

The main limitations of this work are the reduced number of patients and the diversity of TNF blockers studied, which we tried to overcome by studying the same patient before and after therapy.

Taken together with the results found in the literature, these findings suggest that TNFi decrease bone resorption, independently of the control of disease activity. We propose that this is due to the direct reduction of OC classical precursors and inhibition of intracellular signaling pathways involving TRAF6 resulting in a downregulation of CTSK expression and consequent lack of OC motility.

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Supplementary table 5 - Primers used for osteoclast gene expression

Gene	Primer sequence	Annealing temperature	Transcript size
RANK	Fw 5' - GAACATCATGGGACAGAGAAATC - 3' Rev 5' - GGCAAGTAAACATGGGGTTC - 3'	60ºC	89bp
TRAF6	Fw 5' - GCACTAGAACGAGCAAGTGAT - 3' Rev 5' - GGCAGTTCCACCCACACTAT - 3'	60ºC	153bp
FRA-2	Fw 5' - CAGCAGAAATTCCGGGTAGA - 3' Rev 5' - CATGGAGGTGATCACTGTGG - 3'	60ºC	120bp
ATP6V2D0	Fw 5' - CATTCTTGAGTTTGAGGCCG - 3' Rev 5' - CCGTAATGATCCGCTACGTT - 3'	60ºC	186bp
TRAP	Fw 5' - CGGCCACGATCACAATCT - 3' Rev 5' - GCTTTGAGGGGTCCATGA - 3'	60ºC	92bp
CTSK	Fw 5' - GCCAGACAACAGATTTCCATC - 3' Rev 5' - CAGAGCAAAGCTCACCACAG - 3'	60ºC	75bp
18s rRNA	Fw 5' - GGAGTATGGTTGCAAAGCTGA - 3' Rev 5' - ATCTGTCAATCCTGTCCGTGT - 3'	60ºC	129bp

IV. Ankylosing spondylitis patients have impaired osteoclast gene expression in circulating osteoclast precursors

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Inês P. Perpétuo designed the experiments and performed the flow cytometry, cell isolation, cell culture, functional assays, cytokine and bone turnover markers quantification, gene expression, image and statistical analysis, produced the figures and wrote the manuscript.

Ankylosing spondylitis patients have impaired osteoclast gene expression in circulating osteoclast precursors

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Abstract

Introduction: Ankylosing spondylitis (AS) is typically characterized by focal bone overgrowth but also by systemic bone loss. We hypothesize that the increased osteoproliferation found in AS might be partially due to reduced ability of osteoclast precursors (OCPs) to differentiate into osteoclasts (OC). Therefore, our aim was to characterize bone remodeling and pro-osteoclastogenesis inflammatory environment, monocytes phenotype and *in vitro* osteoclast differentiation in AS patients. **Methods:** Patients with active AS without any ongoing therapy and age and gender-matched healthy donors were recruited. Receptor activator of nuclear factor-κβ (RANKL) surface expression on circulating leukocytes and frequency and phenotype of monocyte subpopulations were assessed. Quantification of serum levels of bone turnover markers and cytokines, *in vitro* OC differentiation assay and quantitative reverse transcription real-time PCR for OC specific genes were performed. **Results:** Pro and anti-inflammatory cytokine serum levels were higher in AS patients than in controls. RANKL neutrophil expression was higher in AS patients when compared to healthy donors, but CD51/CD61 expression was lower in the classical monocyte subpopulation. Concerning osteoclastogenesis, we found no differences in the *in vitro* osteoclast differentiating potential of these cells when compared to healthy donors. However, we observed low expression of CSF1R, RANK and NFATc1 in AS osteoclast precursors. **Conclusions:** Despite the high levels of pro-inflammatory cytokines present in AS patients, no differences in the number of OC or resorbed area were found between AS patients and healthy donors. Moreover, we observed that OCPs have low OC specific gene expression. These findings support our hypothesis of an impaired response of OCPs to pro-osteoclastogenic stimuli *in vivo* in AS patients.

Introduction

Ankylosing spondylitis (AS) is an immune mediated chronic inflammatory disease, affecting predominantly the axial skeleton and enthesis. Although its etiopathology is still unknown, tumor necrosis factor (TNF) and interleukin (IL)-17A have been shown to play a central role [1, 2]. In AS, chronic inflammation has a systemic impact on bone, which is reflected by a higher incidence of osteoporosis and increased bone fragility, although spine BMD assessment is difficult due to the presence of syndesmophytes [3]. In fact, in contrast with RA, trabecular bone loss in AS is accompanied by new bone formation at the enthesis sites [4, 5].

The immune and skeletal systems have several regulatory factors in common and immune system cells have a profound influence on bone metabolism, particularly in chronic inflammatory diseases. Monocytes, the circulating osteoclast precursor (OCPs) cells, are phenotypical and functionally heterogeneous and play a critical regulatory role in inflammation and innate immune responses [6, 7]. Monocytes can be divided into three subpopulations based on their expression of CD14 and CD16 surface markers. The classical subset $CD14^{bright}CD16^{-}$ comprises the phagocytic monocytes, the non-classical subset $CD14^{dim}CD16^{+}$ is responsible for cytokine production and T cell activation and the most recently described intermediate subset, $CD14^{bright}CD16^{+}$, which comprises specialized antigen presenting cells [7]. Although monocytes from the classical subpopulation are considered OCPs, all of these subpopulations can differentiate into OC [8, 9].

Receptor activator of nuclear factor κB (RANK) ligand (RANKL) is a key molecule for OC differentiation and activity. RANKL is present on osteoblasts surface, but is also expressed by activated immune cells. These cells contribute indirectly to OC differentiation and function through the secretion of pro-inflammatory cytokines, particularly TNF, IL-1β, IL-6 and IL-17A [2, 10, 11]. Taken together, this environment contributes to increased OC differentiation and, consequently, bone resorption [10, 11].

It has been previously postulated that AS may have an early phase, featured by erosions in sacroiliac joints and enthesis, and a later stage, when the disease course is dominated by new bone formation (syndesmophytes and ankylosis) [4]. Therefore, AS is characterized by exaggerated repair and bone overgrowth [12]. Evidence from animal models suggests that dickkopf related protein (DKK)-1 and sclerostin (SOST), both inhibitors of the Wnt/β-catenin pathway and thus inhibitors of osteoblast function, are

downregulated in AS, resulting in increased osteogenesis [13, 14]. On the other hand, previous studies have found subtle histological differences in the synovial tissue between AS and RA patients, namely the RANKL/osteoprotegerin (OPG) ratio, which was shown to be significantly lower in AS [15]. However, not much attention has been given to the bone resorption features of AS. We have previously shown in a cohort of AS patients before TNF-blocking therapy a decreased *in vitro* differentiation rate of monocytes into OC [16].

In this study we hypothesize that AS OCP have impaired capacity to respond to osteoclastogenic stimuli, irrespectively of inflammation in AS. Therefore, the aim of this study was to characterize bone remodeling and pro-osteoclastogenesis inflammatory environment, monocytes phenotype and *in vitro* OC differentiation in AS patients.

Patients and Methods

Patients

Patients who fulfilled the 1984 New York modified criteria for AS [17] were recruited from the Rheumatology and Bone Metabolic Disease Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. Inclusion criteria included active disease, defined as an AS disease activity score (ASDAS-CRP)>1.3 [18] and documented axial involvement by X-ray or magnetic resonance imaging (MRI). Patients previously exposed or presently under disease modifying anti-rheumatic drugs (DMARDs) or biological DMARDs were excluded. The previous use of non-steroidal anti-inflammatory drugs (NSAIDs) was allowed. Information about gender, age, disease duration, HLA-B27 status, peripheral manifestations and the presence of syndesmophytes was collected. Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured and the ASDAS, the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI [19]) and the Bath Ankylosing Spondylitis Functional Index (BASFI [20]) were assessed. Twenty-one healthy donors matched for age and gender were used as controls.

Heparinized blood from each participant was used for whole blood flow cytometry and peripheral blood mononuclear cell (PBMC) isolation. The Hospital de Santa Maria ethics committee approved this study and all participants signed an informed consent. Patient's management was performed in accordance with the standard practice and this

study was conducted in accordance with the Declaration of Helsinki, as amended in Brazil (2013).

Cytokine detection in the serum

IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-22, interferon gamma (IFN-γ), macrophage inflammatory protein (MIP-1α), transforming growth factor-beta (TGF-β) and TNF levels were measured in the serum by FlowCytomix custom assay kits (Bender MedSystems), according to the manufacturer instructions. Samples were acquired with a FACS Calibur flow cytometer (BD Biosciences). Raw data of the flow cytometry bead assay were analyzed using the FlowCytomix Pro 3.0 software (Bender MedSystems). Carboxy-terminal type I collagen crosslinks (CTX-I, Sunred Biological technology), human type I procollagen amino-terminal-propeptide (P1NP, Sunred Biological technology), OPG, SOST, DKK-1 and soluble RANKL (Biomedica Grouppe) were quantified by ELISA according to the manufacturer's instructions.

Antibodies and flow cytometry

Identification of B and T cells and neutrophils in peripheral blood, and immunophenotyping of monocytes in the PBMC samples, were performed using matched combinations of anti-human murine mAbs. For peripheral blood staining anti-CD19 PerCP-Cy5.5 (eBioscience), anti-CD3 PerCP (BD Biosciences), anti-CD66b FITC (Immunotools) and anti-RANKL PE (Santa Cruz Biotechnology) were used. Monocyte subpopulations were identified with anti-CD14 FITC (BD Biosciences) or PerCPCy5.5 (Immunotools) and anti-CD16 APC (Immunotools) and stained with combinations of anti-CD11b PE-Cy7, CD105 PE, CD62L PE-Cy7, CD51/CD61 FITC (eBioscience), CCR2 PE (R&D Systems), HLA-DR PerCP (BD Biosciences) and RANK PE (Santa Cruz Biotechnology). Cell death was assessed by staining with Annexin V Apoptosis Detection Kit APC (eBioscience). Acquisition was performed using a FACSCalibur (BD Biosciences). Heparinized whole blood was used for staining. Erythrocytes were lysed with red blood cell lysis buffer and cells were incubated with IgG block solution 300ng/mL (ChromPure Mouse IgG whole molecule, Jackson ImmunoResearch Laboratories) before staining. Absolute cell counts were calculated from differential leukocyte count determined for all participants. PBMCs were isolated by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich). Monocyte subpopulations were identified as described before based on their

CD14 and CD16 surface expression [7]. Data was analyzed using FlowJo software (TreeStar, Stanford University).

Osteoclast differentiation

PBMCs isolated by density gradient centrifugation were plated in 96-well culture plates at a density of $7.0x10^5$ cells/well. PBMCs were left overnight for OC precursors to adhere on bone slices and were further cultured for 21 days with M-CSF (25 ng/mL), sRANKL (50 ng/mL), dexamethasone (10 nM) and TGF-β (2.5 ng/mL), as described previously [16, 21, 22]. The culture medium was then changed twice a week. Cells cultured on bone slices for 7, 14 and 21 days [21, 23] were used for functional assays and gene expression.

Functional assays

TRAP staining of OCs was performed using the Acid Phosphate, Leukocyte Kit (TRAP, Sigma-Aldrich) according to the manufacturer's instructions for counting mature OCs (TRAP positive cells containing three or more nuclei). In the resorption assay, to measure the resorbed area, OCs were removed from the bone slices using sodium hypochlorite and stained with 0.1% toluidine blue. Bone slices from both TRAP staining and resorption functional assays were photographed in an area of 1.25 mm^2 with a bright field microscope (Leica DM2500, Leica). The number of TRAP stained OCs was counted for each time-point, per condition, and the resorption pits were traced using ImageJ (NIH, Bethesda, MD). The resorbed area was calculated and expressed in percentage of total area.

Gene expression

RNA was extracted from cells at days 1, 7, 14 and 21 of culture using NZYol (NZYTech). Following RNA extraction, total RNA concentration and purity was quantified using Nanodrop 1000 (Thermo Scientific). Complementary (c)DNA was synthesized at a concentration of 0.6 ng/µL using the DyNAmo™ cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Genes that encode for osteoclast proteins such as CSF1R, RANK, NFATc1, ATP6V0D2 and CTSK were studied (see Supplementary table 1 for primers). Ribosomal RNA 18s was chosen as the housekeeping gene. Primers were designed using the primer-BLAST [24] software and qPCR was performed using the DyNAmo™ Flash SYBR Green qPCR Kit (Thermo

Scientific). The efficiency of qPCR was analysed using the standard curve method, as described previously [25, 26]. The values obtained were normalized with the housekeeping gene 18s rRNA.

Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (IBM, USA). Categorical variables were expressed as frequencies and differences were tested using chi-square test. Continuous variables were expressed by median and interquartile range. The Mann-Whitney non-parametric test was used to compare medians and Spearman correlations were performed. P-values were considered statistically significant when lower than 0.05.

Results

Population characteristics

Thirty AS patients, 11 women and 19 men, with a median age of 40 [33-51] years and ASDAS-ESR of 3.8 [2.4-4.0] were enrolled in this study as well as 21 healthy donors, 8 women and 13 men, with a median age of 45 [36-53] years. All patients presented axial involvement documented by X-ray or MRI, however only 30% of the patients presented syndesmophyte formation. The characteristics of each group are summarized in Table 1.

Pro-inflammatory cytokine levels were higher in AS patients than in controls

All the analyzed cytokines and chemokines had significantly higher circulating levels in AS patients when compared to healthy donors (IL-1β p=0.0001; IL-2 p=0.0005; IL-4 p=0.0211; IL-6 p=0.0005; IL-8 p=0.0001; IL-10 p=0.0353; IL-12p70 p=0.00189; IL-17A p=0.0010; IL-18 p=0.0002; IL-22 p=0.0332; IFN-γ p=0.0005; MIP-1α p=0.0428; TGF-β p=0.0003; TNF p=0.0189; Supplementary table 2). After adjusting for multiple comparisons only IL-1β, IL-2, IL-6, IL-8, IL-17A, IL-18, IFN-γ, TGF-β and TNF remained significantly higher in AS patients as compared to controls. No correlation was found between cytokine levels and disease activity scores (BASDAI or ASDAS). Positive correlations were found between the functional activity index BASFI and the circulating levels of IL-1β (r=0.414, p=0.004), IL-6 (r=0.485, p=0.014), IL-8 (r=0.407, p=0.019), IL-10 (r=0.498, p=0.011), IL17A (r=0.489, p=0.013) and IL-18 (r=0.415, p=0.039) in AS patients.

Table 1 - Characterization of the studied population

	AS patients	Healthy	p-value
Age (years)	40 [33-51]	45 [36-53]	0.4081
Male (%)	63%	62%	1.0000
Symptoms duration (years)	11 [8-21]	NA	
ESR (mm/h)	23 [11-38]	NA	
CRP (mg/dl)	0.8 [0.2-1.7]	NA	
ASDAS-CRP	3.8 [2.4-4]	NA	
BASDAI	5.8 [3.7-7.3]	NA	
BASFI	5.7 [2.1-7.5]	NA	
HLA-B27 (% positive)	77%	NA	
Presence of syndesmophytes (%)	30%	NA	
Peripheral involvement (%)	37%	NA	
Treatment with NSAIDs (%)	60%	NA	
NSAIDs duration (months)	12 [6-52]	NA	

Data is represented as median [interquartile range] unless stated otherwise; NA – not applicable; AS – ankylosing spondylitis; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; ASDAS – ankylosing spondylitis disease activity score; BASDAI – Bath ankylosing spondylitis disease activity index; BASFI – Bath ankylosing spondylitis functional index; HLA – human leukocyte antigen; NSAIDs - non-steroidal anti-inflammatory drugs.

Systemic bone turnover markers were lower in AS patients as compared to healthy controls

Regarding the bone remodeling proteins, no differences in circulating levels of sRANKL, OPG and SOST were found, but DKK-1 was significantly higher in AS patients when compared to healthy donors (p=0.0394; Supplementary table 2 and Fig.1). The levels of CTX-I and P1NP were lower in AS patients when compared to

healthy donors (Fig.1), but no difference in the CTX-I/P1NP ratio was found. After adjusting for multiple comparisons, P1NP and CTX-I remained significantly lower in AS patients. No association was found between bone turnover markers and disease activity scores.

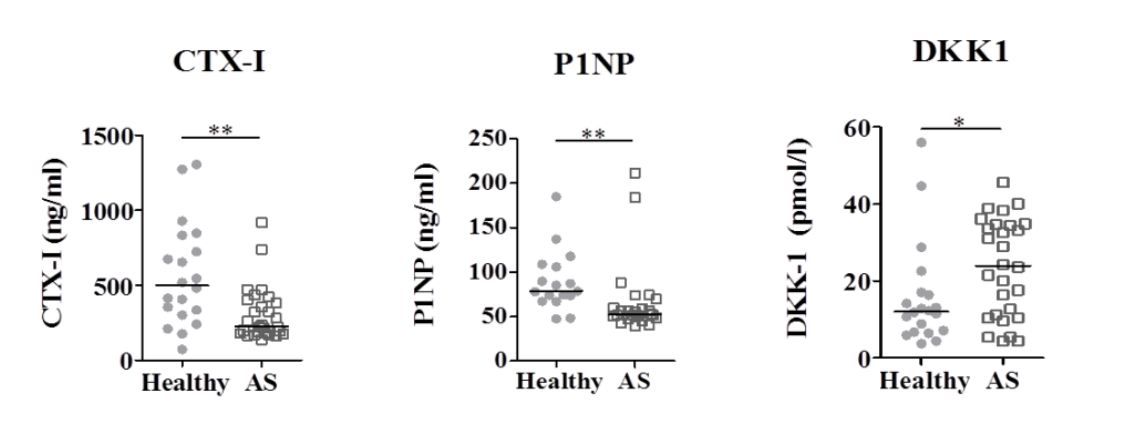

Figure 1. *Serum levels of bone turnover markers and DKK1.* Each dot represents a sample. Line represents median. *p<0.05 and **p<0.01. CTX - carboxy-terminal telopeptide of type I collagen, P1NP - total procollagen type 1 N-terminal propeptide, DKK1 – dickkopf related protein 1.

RANKL neutrophil expression was higher in AS patients when compared to healthy controls

The frequency of neutrophils, B and T lymphocytes was analyzed, as well as RANKL surface expression in these cells. The number of neutrophils was higher in AS patients when compared to the control group (p=0.0059; Fig.2A). Regarding RANKL surface expression a higher number and percentage of RANKL expressing neutrophils in AS patients was found when compared to healthy donors (p=0.0022 and p=0.0071, respectively); however, surface RANKL was lower on these cells (p=0.0134) when compared to controls. No differences were observed in the total circulating T or B lymphocytes (percentage and total number), in RANKL surface expression or in cell death between any of the studied populations.

CD51/CD61 (αvβ3 integrin) surface expression was lower in osteoclast precursors from AS patients

Monocyte subpopulations were analyzed after PBMC isolation and staining. The frequency of non-classical $CD14^{dim}CD16^{+}$ monocytes was lower in AS patients when

compared to control donors (p=0.0490; Fig.2B). No differences were found between groups in the intermediate $CD14^{bright}CD16^{+}$ or in the classical $CD14^{brigth}CD16^{-}$ (data not shown) subpopulations. Spearman correlation showed that, in AS patients, the frequency of classical and non-classical subpopulations was inversely correlated (r=-0.948, p<0.001).

Regarding the phenotype of circulating monocytes, in AS patients, the classical subpopulation had lower surface expression of CD51/CD61 (αvβ3 integrin) (p=0.0074; Fig.2C). The intermediate subpopulation had a higher expression of CD51/CD61 and RANK (p=0.0061 and p=0.0011 respectively) and the non-classical subpopulation had more CD62L surface expression (p=0.0457), when compared to healthy controls. No differences in cell death between groups were found (data not shown).

Osteoclast differentiating genes were downregulated in osteoclast precursors from AS patients

Osteoclast number increased from culture day 7 to 21. Although differences were not found between groups, at culture day 21, AS patients had less OCs than healthy donors. The number of nuclei per OC was similar between groups. Pit number and percentage of resorption area markedly increased after day 14 in both groups; however, no differences in the resorption activity of these cells or in the pit size were found between groups (Fig.3A and B).

Regarding OC gene expression, CSF1R, RANK and NFATc1 were significantly downregulated at day 1 in AS patients, when compared to healthy donors (Fig.3C). On the last culture day CSF1R, NFATc1, ATP6V0D2 and CTSK expression was significantly reduced in cells from AS patients. After correcting for multiple comparisons ATP6V0D2 and CTSK expression at culture day 21 remained significantly reduced in cells from AS patients.

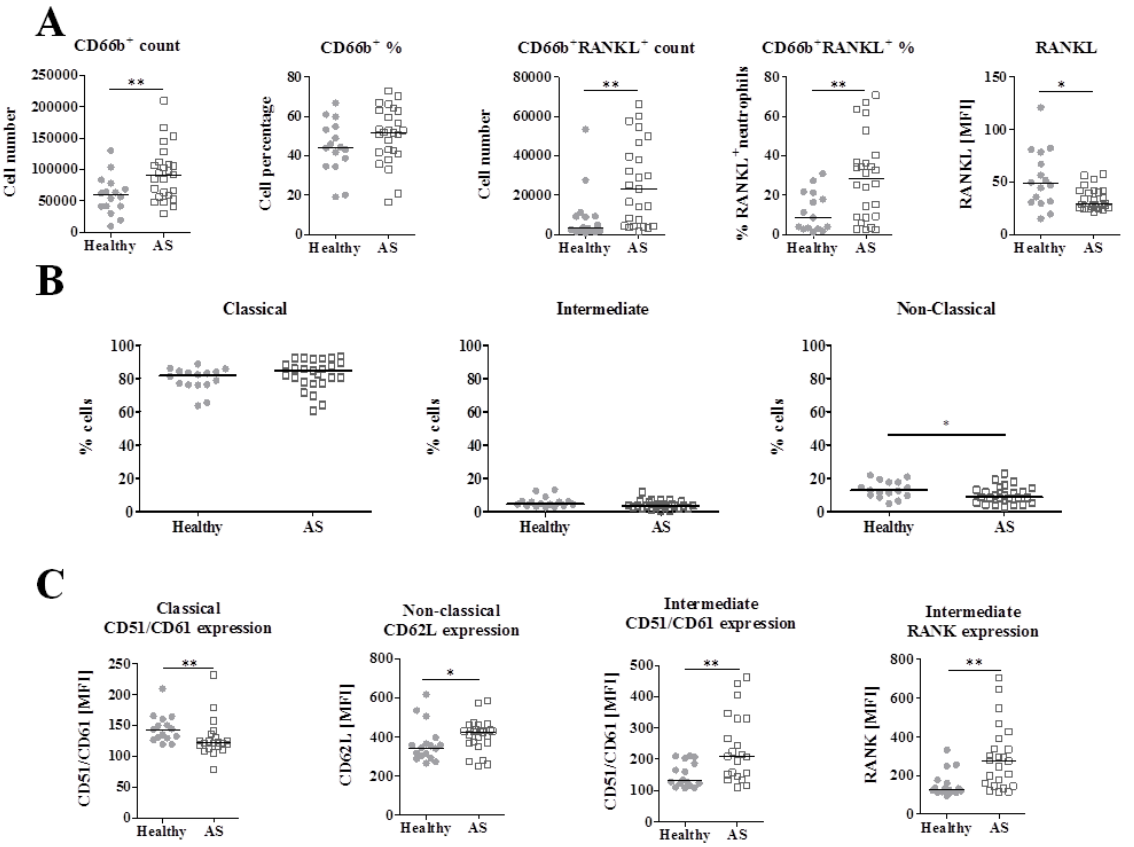

Figure 2. *Leukocyte analysis by flow cytometry.* A) $CD66b^+$ circulating cells are increased in AS patients when compared to healthy donors (p=0.0059 for absolute number count). $CD66b^+RANKL^+$ cells are increased in the circulation of AS patients when compared to healthy donors (both in absolute number and in percentage, p=0.0022 and p=0.0071 respectively). However RANKL expression on neutrophils surface is significantly decreased in AS patients when compared to healthy donors (p=0.0134). No differences were observed for T or B lymphocytes in circulation. B) The frequency of the non-classical monocyte subpopulation ($CD14^{dim}CD16^+$) is decreased in AS patients when compared to healthy donors (p=0.0490). No differences were found in the circulating classical or intermediate subpopulations. C) Phenotype of circulating monocyte subpopulations; CD51/CD61 (αVβ3 integrin) in decreased in the classical subpopulation in AS patients (p=0.0074), however it is increased in the intermediate subpopulation of AS patients when compared to healthy donors (p=0.0061); CD62L (L-selectin) is increased in the non-classical subpopulation of AS patients when compared to healthy donors (p=0.0457) and RANK expression is increased in the intermediate subpopulation of AS patients when compared to healthy donors (p=0.0011). Each dot represents a sample. Line represents median. P<0.05 is considered significant. *p<0.05 and **p<0.01. MFI - Median fluorescence intensity.

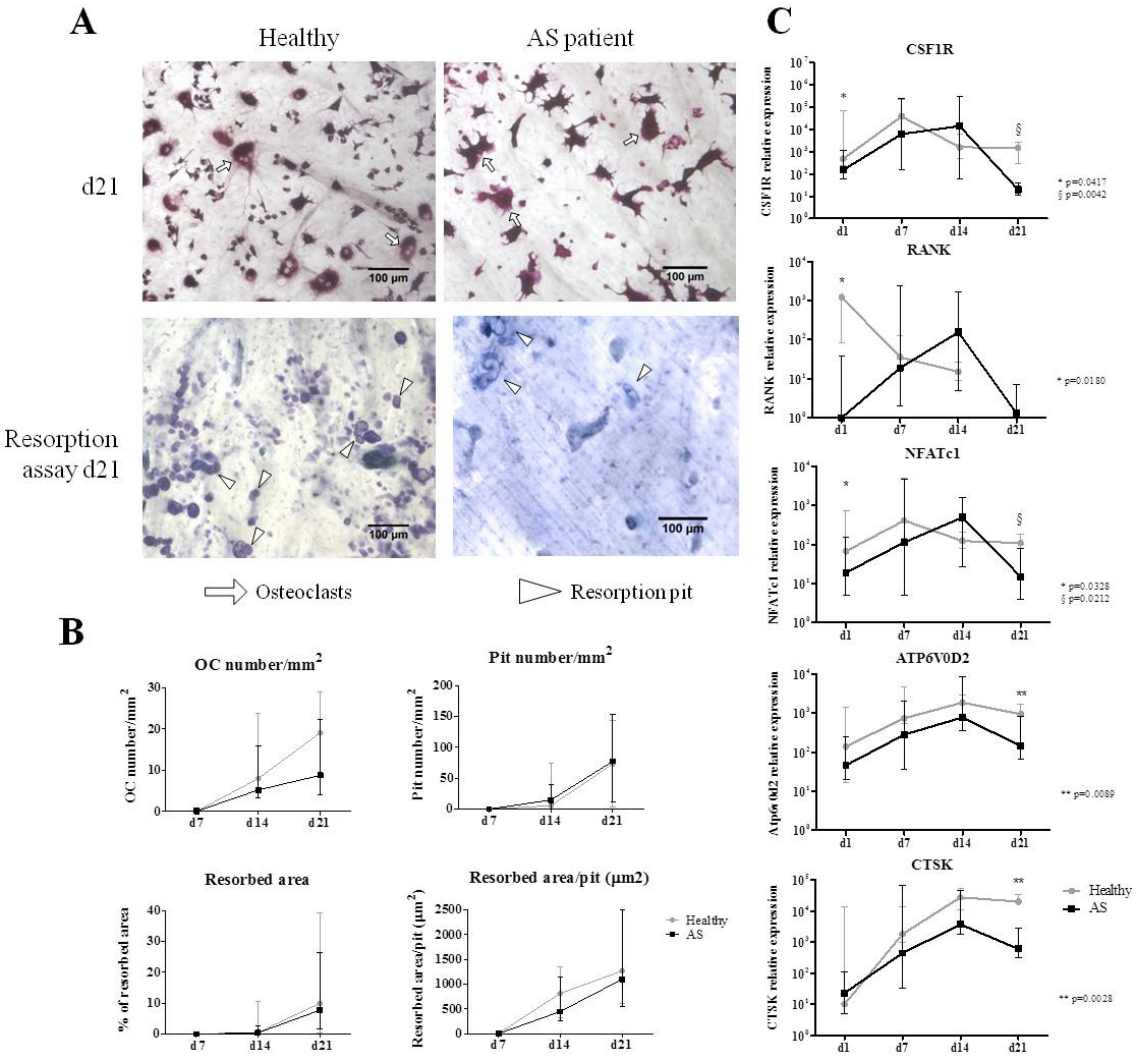

Figure 3. *Functional assays and osteoclast gene expression.* A) Representative images of TRAP staining and resorption pits at culture day 21 are shown for healthy and AS patients. 10x magnification. White arrows - osteoclasts; white arrowheads - resorption pit. B) Osteoclast number, resorbed area, pit number and resorbed area/pit increased throughout time without significant differences between groups. C) Gene expression profile of cells in culture for 21 days. CSF1R, RANK and NFATc1 are significantly decreased at day 1 in cells from AS patients when compared to healthhy donors. We found no differences between groups throughout the culture time except at culture day 21 where all genes are significantly higher in cells differentiated from healthy donors except for RANK. Relative gene expression shown in Log scale. Dots in graphs represent median gene expression for each group at each time-point and bars represent interquartile range. d - day, OC - osteoclast, CSF1R - gene encoding macrophage-colony stimulating factor (c-fms), RANK - gene encoding for receptor activator of nuclear factor-κβ, NFATc1 - gene encoding for nuclear factor of activated T-cells, Atp6v0d2 - gene encoding ATPase, H^+ transporting, lysosomal V0 subunit D2, CTSK - gene encoding cathepsin K. $p<0.05$ is considered significant.

Discussion

We hypothesized that AS OC precursors have impaired capacity to respond to osteoclastogenic stimuli, regardless of inflammation in AS. In this study we characterized the bone remodeling and pro-osteoclastogenic inflammatory environment, monocyte phenotype and *in vitro* OC differentiation of OCP in AS patients without any disease modifying therapy.

Serum levels of IL-1β, IL-2, IL-6, IL-8, IL-17A, IL-18, IFNγ, TGF-β and TNF were significantly higher in AS patients when compared to controls, as described before [1, 27-30]. As previously described, the majority of these cytokines are pro-inflammatory and pro-osteoclastogenic [1, 2, 8, 10, 28, 29, 31]. Of interest, TGF-β plays an anti-inflammatory role and is also involved in wound healing and fibrosis [32] and it has been described as an important cytokine for bone formation [33].

SOST and DKK1 are endogenous inhibitors of the Wnt/β-catenin pathway; these proteins are mainly secreted by osteocytes and inhibit osteoblasts differentiation [34]. SOST serum levels were not significantly different between AS and controls, but DKK-1 was significantly higher. Studies assessing levels of DKK-1 and SOST in AS are scarce and have provided conflicting results [14, 31, 35]. Taylan *et al* described that serum levels of DKK-1 and SOST were not different in AS patients with mild to active disease when compared to healthy controls [27, 31]. In contrast, other studies reported lower levels of circulating SOST and of DKK-1 in AS patients when compared to healthy donors. Our results are consistent with a study from Miceli-Richard and colleagues that found no differences between serum levels of SOST when comparing AS patients with healthy donors [35].

When looking at the RANKL/OPG axis, no differences in sRANKL, OPG or sRANKL/OPG ratio were found between groups. Previous studies have shown discrepancies in sRANKL and OPG serum levels in AS patients. In some studies sRANKL and OPG have been found to be increased in AS patients with active disease [36]; however, in other studies AS patients with mild to active disease have lower sRANKL/OPG [31, 37] than controls. Different results for sRANKL measurements might be due to different patient's characteristics, sample size and also to the use of different detection methods.

CTX-I and P1NP levels were lower in AS patients when compared to healthy controls, reflecting a low bone turnover. However, recent reports have shown increased levels of

urinary CTX-I levels in AS patients when compared to healthy donors [38]. Bone turnover markers values remain highly controversial in the literature, since they are also affected by time of collection and circadian rhythms [39, 40].

Circulating leukocyte numbers and RANKL expression was analyzed by flow cytometry. We showed that there was an increase in the total number of neutrophils as previously shown by several studies in AS patients [41-44]. We also observed higher numbers of $RANKL^+$ neutrophils, despite the lower surface expression of this protein, as compared to controls. It was previously shown that activated neutrophils express RANKL on their surface and are capable of promoting osteoclastogenesis in other rheumatic diseases and healthy donors [45, 46].

Monocytes can be classified into three subpopulations that are phenotypically and functionally heterogeneous. The classical subpopulation is the most abundant subset and includes the OCP. Although the function of monocytes is well characterized in inflammation, their role in AS axial disease is not clear [6, 7]. CD51/CD61 ($\alpha_V\beta_3$ integrin) is important for monocyte and OC adhesion to the bone matrix and for providing survival signals and mobility traits. According to our results, CD51/CD61 expression was lower in the classical subpopulation of AS patients and in the intermediate subpopulation both CD51/CD61 and RANK expression were higher, in comparison to controls. To the best of our knowledge this is the first time that the phenotype of the previously described monocyte subpopulations [7] has been addressed in AS patients. The decreased CD51/CD61 expression in the classical monocyte subpopulation, which has traditionally been regarded as the OC precursor, may be a clue for the pattern of bone resorption seen in AS. This observation is consistent with the study performed by Gengenbacher and colleagues that showed lower bone resorption *in vitro* in AS when compared to RA patients, although the different monocytes subpopulations were not assessed [47].

No differences were found in the number of OCs, the number of resorption pits or the resorbed area. However, OC specific gene expression was significantly lower in cells from AS patients when compared to healthy donors. This was observed not only at culture day 1 for CSF1R, RANK and NFATc1, but also at day 21 for CSF1R, NFATc1, ATP6V0D2 and CTSK. We believe that this decrease in OC specific genes expression in the circulating precursors and the significant decrease of the expression of genes that encode bone degrading proteins at culture day 21 are indicators of reduced osteoclastogenesis in AS, probably due to poor response to osteoclastogenic stimuli.

High levels of pro-inflammatory cytokines and increased surface RANKL expression have been previously associated with increased OC differentiation and activity. According to our study, despite high levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-17A and TNF) and of high number of RANKL-expressing neutrophils, no differences were found in OC number or activity between AS patients and healthy controls. Moreover, osteoclast specific gene expression was significantly lower in cells from AS patients when compared to healthy donors. We believe that this decrease in osteoclast specific genes expression in the circulating precursors and the significant decrease of the expression of genes that encode bone degrading proteins at culture day 21 are indicators of reduced osteoclastogenesis in AS.

We propose that the low RANK and CD51/CD61 expression in classical monocytes contribute to decrease OC formation and adhesion to bone, resulting in reduced capacity of these cells to resorb bone.

Although chronic inflammatory diseases like RA and psoriatic arthritis are classically associated with increased bone resorption [48-52], and regardless of the pro-inflammatory environment found in AS, our data shows decreased indicators of osteoclast formation and bone resorption suggesting reduced capacity of OCPs to differentiate into osteoclasts and resorb bone.

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Supplementary table 1 - Primers used for osteoclast gene expression

Gene	Primer sequence	Transcript size
CFSR1	Fw 5' - GAACATCCACCTCGAGAAGAAA - 3' Rev 5' - GACAGGCCTCATCTCCACAT - 3'	88 bp
RANK	Fw 5' - GAACATCATGGGACAGAGAAATC - 3' Rev 5' - GGCAAGTAAACATGGGGTTC - 3'	89 bp
NFATc1	Fw 5' - GCAAGCCGAATTCTCTGGTG - 3' Rev 5' - TACCGTTGGCGGGAAGGTAG - 3'	144 bp
ATP6V2D0	Fw 5' - CATTCTTGAGTTTGAGGCCG - 3' Rev 5' - CCGTAATGATCCGCTACGTT - 3'	186 bp
CTSK	Fw 5' - GCCAGACAACAGATTTCCATC - 3' Rev 5' - CAGAGCAAAGCTCACCACAG - 3'	75 bp
18s rRNA	Fw 5' - GGAGTATGGTTGCAAAGCTGA - 3' Rev 5' - ATCTGTCAATCCTGTCCGTGT - 3'	129 bp

All primers had an annealing temperature of 60ºC

Table 2 - Serum cytokine and bone turnover markers levels

	AS patients	Healthy	p-value
IL-1β (pg/ml)	454 [4 - 4783]	4 [4 - 4]	**0.0001** †
IL-2 (pg/ml)	3855 [16 - 5703]	16 [16 - 16]	**0.0005** †
IL-4 (pg/ml)	10796 [21 - 19082]	21 [21 - 639]	**0.0211**
IL-6 (pg/ml)	15.8 [1.2 - 478.3]	1.2 [1.2 - 1.2]	**0.0005** †
IL-8 (pg/ml)	161 [25 - 927]	0.5 [0.5 - 0.5]	**0.0001** †
IL-10 (pg/ml)	12 [2 - 8547]	2 [2 - 1110]	**0.0353**
IL-12p70 (pg/ml)	1.5 [1.5 - 1163.9]	1.5 [1.5 - 132.3]	**0.0189**
IL-17A (pg/ml)	1412 [2.5 - 6469]	2.5 [2.5 - 2.5]	**0.0010** †
IL-18 (pg/ml)	13199 [812 - 22608]	3.3 [3.3 - 3066]	**0.0002** †
IL-22 (pg/ml)	4354 [1742 - 7323]	1624 [43 - 3586]	**0.0332**
IFN-γ (pg/ml)	4013 [2 - 16485]	2 [2 - 2]	**0.0005** †
MIP-1α (pg/ml)	666 [1 - 5958]	1 [1 - 3904]	**0.0428**
TGF-β (pg/ml)	6333 [2866 - 28798]	2219 [1478 - 2753]	**0.0003** †
TNF (pg/ml)	574 [3 - 8129]	3 [3 - 3]	**0.0189** †
CTX-I (ng/ml)	228 [181 - 297]	500 [310 - 807]	**0.0025** †
P1NP (ng/ml)	52 [50 - 62]	79 [71 - 107]	**0.0012** †
CTX-I/P1NP	3.7 [3.4 - 5.5]	5.9 [3.0 - 7.3]	0.3724
sRANKL (pmol/L)	0.02 [0.02 - 0.18]	0.02 [0.02-0.25]	0.6273
OPG (pmol/L)	3.1 [2.6 - 4.2]	3.1 [2.4 - 3.6]	0.6084
sRANKL/OPG	0.009 [0.004 - 0.076]	0.009 [0.006 - 0.070]	0.9917
SOST (pmol/L)	27 [23 - 33]	29 [23 - 36]	0.6439
DKK1 (pmol/L)	24 [11 - 35]	12 [7 - 17]	**0.0394**

Data is represented as median [interquartile range]. p-values in bold are statistically significant. † - values remain significant after adjusting for multiple comparisons. AS - ankylosing spondylitis, IL - interleukin, IFN - interferon, MIP - macrophage inflammatory protein, TGF - transforming growth factor, TNF - tumor necrosis factor, CTX-I - carboxy-terminal collagen crosslinks, P1NP - procollagen type 1 amino-terminal propeptide, sRANKL - soluble receptor activator of nuclear factor κB, OPG - osteoprotegerin, SOST - sclerostin, DKK1 - dickkopf-related protein 1 .

V. Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Ankylosing Spondylitis.

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*Inês P. Perpétuo and Rita Raposeiro equally contributed to this work

Inês P. Perpétuo designed all the experiments and performed the flow cytometry and cell isolation. Cell culture, functional assays, cytokine and bone turnover marker quantification, gene expression, image and statistical analysis, figure production were performed in collaboration with Rita Raposeiro. Inês P. Perpétuo wrote the manuscript.

Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Ankylosing Spondylitis

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Abstract

Introduction: Ankylosing Spondylitis (AS) is characterized by excessive local bone formation and concomitant systemic bone loss. Tumor necrosis factor (TNF) plays a central role in the inflammation of axial skeleton and enthesis of AS patients. Despite reduction of inflammation and systemic bone loss, AS patients treated with TNF inhibitors (TNFi) have ongoing local bone formation. The aim of this study was to assess the effect of TNFi in the differentiation and activity of osteoclasts (OC) in AS patients. **Methods:** 13 AS patients treated with TNFi were analyzed at baseline and after a minimum follow-up period of 6 months. 25 healthy donors were recruited as controls. Blood samples were collected to assess receptor activator of nuclear factor kappa-B ligand (RANKL) surface expression on circulating leukocytes and frequency and phenotype of monocyte subpopulations. Quantification of serum levels of bone turnover markers and cytokines, *in vitro* OC differentiation assay and qRT-PCR for OC specific genes were performed. **Results:** $RANKL^+$ circulating lymphocytes (B and T cells) and IL-17A, IL-23 and TGF-β levels were decreased after TNFi treatment. We found no differences in the frequency of the different monocyte subpopulations, however, we found decreased expression of CCR2 and increased expression of CD62L after TNFi treatment. OC number was reduced in patients at baseline when compared to controls. OC specific gene expression was reduced in circulating OC precursors

after TNFi treatment. However, when cultured in OC differentiating conditions, OC precursors from AS TNFi-treated patients showed increased activity as compared to baseline. **Conclusion:** In AS patients, TNFi treatment reduces systemic pro osteoclastogenic stimuli. However, OC precursors from AS patients exposed to TNFi therapy have increased *in vitro* activity in response to osteoclastogenic stimuli.

Introduction

Ankylosing spondylitis (AS) is a systemic, chronic, immune-mediated inflammatory disease that affects the musculoskeletal system. The axial skeleton and enthesis are predominantly involved in this disease and tumor necrosis factor (TNF) seems to play a central role [1]. AS is characterized by local excessive bone formation, but it is also associated with systemic bone loss, which is a common complication even in the early stages of the disease [2].

The immune and skeletal systems have several common regulatory factors and immune system cells have a profound influence on bone metabolism, particularly in chronic inflammatory diseases. Receptor activator of nuclear factor κB ligand (RANKL) is present on osteoblasts surface, but is also expressed by activated immune cells, both in its membrane form and as a soluble molecule [3]. Cytokines such as TNF, interleukin (IL)-1β, IL-6 and IL-17 are secreted by activated immune cells and act synergistically with the RANK-RANKL system [4, 5], further enhancing osteoclast (OC) differentiation from its circulatory precursors (monocytes) and contributing to bone resorption [1, 3]. Monocytes are phenotypically and functionally heterogeneous and have a critical regulatory role in inflammation and innate immune responses [6]. Three sub-populations of monocytes have been described in humans, based on their expression of CD14 and CD16 surface markers. The classical subset, $CD14^{bright}CD16^{-}$ accounts for 85% of monocytes, includes phagocytic cells and OC precursors; the non-classical subset $CD14^{dim}CD16^{+}$ accounts for 10% of monocytes and is

involved in cytokine production and T-cell activation. The intermediate, the most recently described subset, accounts for only 5% of monocytes and is $CD14^{bright}CD16^{+}$. This latter subset is considered to be the antigen presenting subset and is responsible for reactive oxygen species production [6]. Monocytes are key players in immune-mediated inflammatory diseases and their excessive and sustained activity is a hallmark of AS [7].

Serum levels of TNF, IL-6 and IL-17 are increased in AS patients, which may contribute to the well documented secondary osteoporosis that occur in these patients [1, 8]. TNFi are very effective in the mitigation of inflammation in AS patients and induce a reduction in CTX-I levels, which may reflect a decrease in OC activity [8]. The aim of this study was to assess the effect of TNFi in the differentiation and activity of OC precursors in AS patients.

Patients and Methods

Patients

The local ethics committee (Hospital de Santa Maria) approved this study and all participants signed an informed consent. Patients were managed in accordance with the standard practice and the study was conducted in accordance with the Declaration of Helsinki as amended in Brazil (2013). Patients with AS fulfilling the New York modified criteria 1984 [9] were recruited from the Rheumatology and Bone Metabolic Disease Department, Lisbon Academic Medical Centre, Portugal. All patients were included before starting the first TNFi and were followed-up during a minimum period of 6 months after initiating therapy. Other inclusion criteria at baseline were: active disease, defined as AS disease activity score (ASDAS-ESR)>1.3 [10] and documented axial involvement by X-ray or magnetic resonance imaging (MRI). Patients previously exposed to biological TNFi were excluded. Information regarding patients' demographics, duration of symptoms, peripheral involvement, syndesmophyte formation, HLA-B27 positivity, erythrocyte sedimentation rate (ESR) and C-reactive protein

(CRP) was collected. ASDAS was evaluated, as well as the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI [11]) and the Bath Ankylosing Spondylitis Functional Index (BASFI [12]). Heparinized blood and serum were collected from each participant at the starting date of TNFi and another collection was made after a minimum period of 6 months of follow-up. Blood was used for flow cytometry and peripheral blood mononuclear cell (PBMC) isolation. Donors matched for age and gender were used as controls. Samples were stored and managed by the Biobanco-IMM, Lisbon Academic Medical Center, Lisbon, Portugal. The local ethics committee approved this study and all participants signed an informed consent.

Flow cytometry

Identification of B and T cells and granulocytes in peripheral blood and immunophenotyping of monocytes in the PBMC samples were performed using matched combinations of anti-human murine mAbs. For peripheral blood staining anti-CD19 PerCP-Cy5.5 (eBioscience), anti-CD3 PerCP (BD Biosciences), anti-CD66b FITC (Immunotools) and anti-RANKL PE (Santa Cruz Biotechnology) were used. Monocyte subpopulations were identified with anti-CD14 FITC (BD Biosciences) or PerCPCy5.5 (Immunotools) and anti-CD16 APC (Immunotools) and stained with combinations of anti-CD11b PE-Cy7, CD105 PE, CD62L PE-Cy7, CD51/CD61 FITC (eBioscience), CCR2 PE (R&D Systems), HLA-DR PerCP (BD Biosciences) and RANK PE (Santa Cruz Biotechnology). Cell death was assessed by staining with Annexin V Apoptosis Detection Kit APC (eBioscience). Acquisition was performed using a FACSCalibur (BD Biosciences).

Heparinized whole blood was used for staining. Erythrocytes were lysed with red blood cell lysis buffer and cells were incubated with IgG block solution 300ng/mL (ChromPure Mouse IgG whole molecule, Jackson ImmunoResearch Laboratories) before staining. Absolute cell counts were calculated from differential leukocyte count determined for all participants.

PBMCs were isolated by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich). Monocyte subpopulations were identified as described before based on their CD14 and CD16 surface expression [6]. Data was analyzed using FlowJo software (TreeStar, Stanford University).

Cytokine detection in the serum

IL-1β, IL-6, IL-12(p70), IL-17A, IL-23, monocyte chemotactic protein-1 (MCP-1), transforming growth factor-beta (TGF-β) and TNF levels were measured in the serum by FlowCytomix custom assay kits (Bender MedSystems) according to the manufacturer instructions. Samples were acquired with a FACS Calibur flow cytometer (BD Biosciences). Raw data of the flow cytometry bead assay were analyzed by FlowCytomix Pro 3.0 software (Bender MedSystems). Carboxy-terminal type I collagen crosslinks (CTX-I), human type I procollagen amino-terminal-propeptide (P1NP, Sunred Biological technology), osteoprotegerin (OPG), sclerostin (SOST), dickkopf-related protein (DKK)-1 and soluble RANKL (ampli-sRANKL, Biomedica Grouppe) were quantified by enzyme-linked immunosorbent assay (ELISA) in serum samples according to the manufacturer's instructions.

PBMC isolation and cell culture

PBMCs were isolated by density gradient centrifugation and plated in 96-well culture plates at a density of $7.0x10^5$ cells/well and in 24-well culture plates at a density of $1.5x10^6$ cells/well in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5000 U Penicilin/Streptomicin (Invitrogen), 2 mM L-Glutamine (Invitrogen) and 10% Fetal Bovine Serum (FBS; Invitrogen) and incubated in a humidified atmosphere at 37ºC, 5% CO_2. PBMCs were left overnight for OC precursors (OCPs) to adhere on bone slices. On the following day (day 1 of culture) medium was changed to DMEM supplemented with M-CSF

25 ng/mL (Peprotech) and three days later, medium was again changed to DMEM supplemented with M-CSF (25 ng/mL), sRANKL (50 ng/mL; Peprotech), dexamethasone (10 nM; Sigma Aldrich) and TGF-β (2.5 ng/mL; R&D Systems) in order to differentiate the osteoclast precursors into mature osteoclasts. The culture medium was then changed twice a week. Adherent cells at day 1 and cells cultured on bone slices for 7, 14 and 21 days [13] were used for functional assays and gene expression.

Functional assays

Tartrate-resistant acid phosphatase (TRAP) staining of OCs was performed at days 7, 14 and 21 of culture using the Acid Phosphate Leukocyte Kit (TRAP, Sigma-Aldrich) according to the manufacturer's instructions. OCs were counted as TRAP positive cells containing three or more nuclei [14, 15]. For measurement of resorbed area in the resorption assay at days 7, 14 and 21 of culture, cells were removed from the bone slices using sodium hypochlorite and stained with 0.1% toluidine blue [16]. Bone slices from both TRAP staining and resorption functional assays were photographed in an area of 1.25 mm^2 with a brightfield microscope (Leica DM2500, Leica) under a 10x objective. The number of TRAP stained OCs was counted for each time-point per condition and the resorption pits were traced using ImageJ software (NIH, Bethesda, MD). The resorbed area was calculated and expressed in % of total area.

Gene expression

RNA was extracted from cells cultured over the bone slices at days 1, 7, 14 and 21 of culture using NZYol (NZYTech) according to the manufacturer's instructions. Following RNA extraction, total RNA concentration and purity was quantified using Nanodrop 1000 (Thermo Scientific). Complementary (c)DNA was synthesized at a concentration of 0.6 ng/µL using the DyNAmo™ cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's

instructions. Genes that encode for osteoclast proteins such as CSF1R, RANK, NFATc1, ATP6V0D2 and CTSK were studied (see Table 1 for primers) by real-time quantitative PCR (RT-qPCR). Ribossomal RNA 18s was chosen as the housekeeping gene. Primers were designed using the primer-BLAST [17] software and qPCR was performed using the DyNAmo™ Flash SYBR Green qPCR Kit (Thermo Scientific). The efficiency of qPCR was analysed using the standard curve method [18] as described previously [19]. The values obtained were normalized with the housekeeping gene 18s rRNA.

Table 1. Primers used for osteoclast gene expression

Gene	Primer sequence	Annealing temperature	Transcript size
CFSR1	Fw 5' - GAACATCCACCTCGAGAAGAAA - 3' Rev 5' - GACAGGCCTCATCTCCACAT - 3'	60ºC	88bp
RANK	Fw 5' - GAACATCATGGGACAGAGAAATC - 3' Rev 5' - GGCAAGTAAACATGGGGTTC - 3'	60ºC	89bp
NFATc1	Fw 5' - GCAAGCCGAATTCTCTGGTG - 3' Rev 5' - TACCGTTGGCGGGAAGGTAG - 3'	60ºC	144bp
ATP6V2D0	Fw 5' - CATTCTTGAGTTTGAGGCCG - 3' Rev 5' - CCGTAATGATCCGCTACGTT - 3'	60ºC	186bp
CTSK	Fw 5' - GCCAGACAACAGATTTCCATC - 3' Rev 5' - CAGAGCAAAGCTCACCACAG - 3'	60ºC	75bp
18s rRNA	Fw 5' - GGAGTATGGTTGCAAAGCTGA - 3' Rev 5' - ATCTGTCAATCCTGTCCGTGT - 3'	60ºC	129bp

Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (IBM). Categorical variables were expressed as frequencies and comparisons were tested using chi-square test. Continuous variables were expressed by median and interquartile range. Baseline and post-treatment (follow-up) values within each sample were compared using Wilcoxon's matched-pairs signed-rank test. To compare AS patients with healthy age and sex-matched donors Mann-Whitney test was used. Correlation analysis was performed using Spearman's correlation coefficients. Values were corrected for multiple comparisons and p-values lower than 0.05 were considered significant.

Results

Patient background

Thirty-eight subjects were recruited, including 13 AS patients, evaluated before and after TNFi therapy, and 25 age and gender matched healthy donors. Despite having an initial cohort of 25 patients 3 were lost for follow-up and 9 switched biological therapy at 3 months follow-up. Patients were treated with one of the four TNFi currently used in clinical practice: Adalimumab (n=1, 8%), Golimumab (n=5, 38.5%), Infliximab (n=2, 15%) or Etanercept (n=5, 38.5%). Treatment duration ranged from a minimum of 6 up to 12 months. The clinical and demographic characteristics of the patients both at baseline and follow-up and healthy donors are described in Table 2.

Table 2. Summary of the patients and healthy controls' characteristics

	AS patients		Healthy	p-value
	Baseline	Follow-up		
Sample size	13		25	
Age (years)	37 [33-43]		39 [36-49]	0.8028
Females %	38%		48%	0.7342
Symptoms duration (years)	10 [7-21]		NA	
HLA-B27 (% positive)	54%		NA	
Presence of syndesmophytes (%)	40%		NA	
Peripheral involvement (%)	46%		NA	
Treatment with NSAIDs (%)	77%		NA	
NSAIDs duration (months)	24 [8-42]		NA	
Treatment with DMARDs (%)	46%		NA	
DMARDs duration (months)	24 [7-47]		NA	
Treatment with corticosteroids (%)	15%		NA	
ESR (mm/h)	30 [14-54]	7 [4-17]	NA	0.0010*
CRP (mg/dl)	1.4 [0.1-3.0]	0.1 [0.0-0.6]	NA	0.0034*
ASDAS	3.8 [2.2-4.3]	1.7 [1.4-1.9]	NA	0.0001*
BASDAI	4.7 [3.9-7.5]	2.5 [1.5-4.1]	NA	0.0007*
BASFI	6.2 [5.1-7.4]	3.9 [1.2-5.4]	NA	0.0032*
TNFi duration (months)	NA	12 [6-12]	NA	

Data is represented as median [Interquartile range] unless stated otherwise; DMARDs include methotrexate, hydroxychloroquine and sulfasalazine; AS – ankylosing spondylitis; HLA – human leukocyte antigen; NA – not applicable; NSAIDs - non-steroidal anti-inflammatory drugs; DMARDs – disease-modifying antirheumatic drugs; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; ASDAS – ankylosing spondylitis disease activity score; BASDAI – Bath ankylosing spondylitis disease activity index; BASFI – Bath ankylosing spondylitis functional index; TNFi – tumor necrosis factor inhibitors. * p-value<0.05.

TNFi treatment in AS patients decreases the number of RANKL$^+$ T and B cells in circulation

RANKL surface staining was performed in whole blood leukocytes (neutrophils - CD66b$^+$; T cells CD3$^+$; B cells CD19$^+$; Fig.1A). No difference was found in the total number of circulating neutrophils, T or B cells before or after therapy or when compared to healthy donors (data not shown). However, CD66b$^+$RANKL$^+$ cells were higher in patients than in healthy donors, both at baseline and follow-up. After TNFi therapy patients had lower number of CD3$^+$RANKL$^+$ cells in circulation when compared to healthy donors, both in percentage and absolute number (p=0.0271 and p=0.0244, respectively; Fig.1A). Furthermore CD19$^+$RANKL$^+$ cell frequency and absolute number was decreased in patients after TNFi treatment (percentage value significantly different, p=0.0122; Fig.1A).

Regarding monocyte subpopulations (classical CD14^{bright}CD16$^-$, intermediate CD14^{bright}CD16$^+$ and non-classical CD14^{dim}CD16$^+$) no differences in frequency or cell death among the three subpopulations and between groups were found. CD62L, a cell adhesion molecule also known as L-selectin, was increased in the circulating classical subpopulation of patients, both at baseline and follow-up, when compared to healthy donors (p=0.0436; p=0.0158; Fig 1B). Moreover, comparing with healthy donors, CD62L expression was higher in patients after 6 months of TNFi therapy both in the non-classical subpopulation (p=0.0398; Fig 1B) and in the intermediate (p=0.001; Fig 1B) subpopulations. Conversely, CCR2 expression was lower in the intermediate subpopulation in patients after TNFi when compared to healthy donors (p=0.0307; Fig 1B). No differences were identified in any of the other studied surface markers.

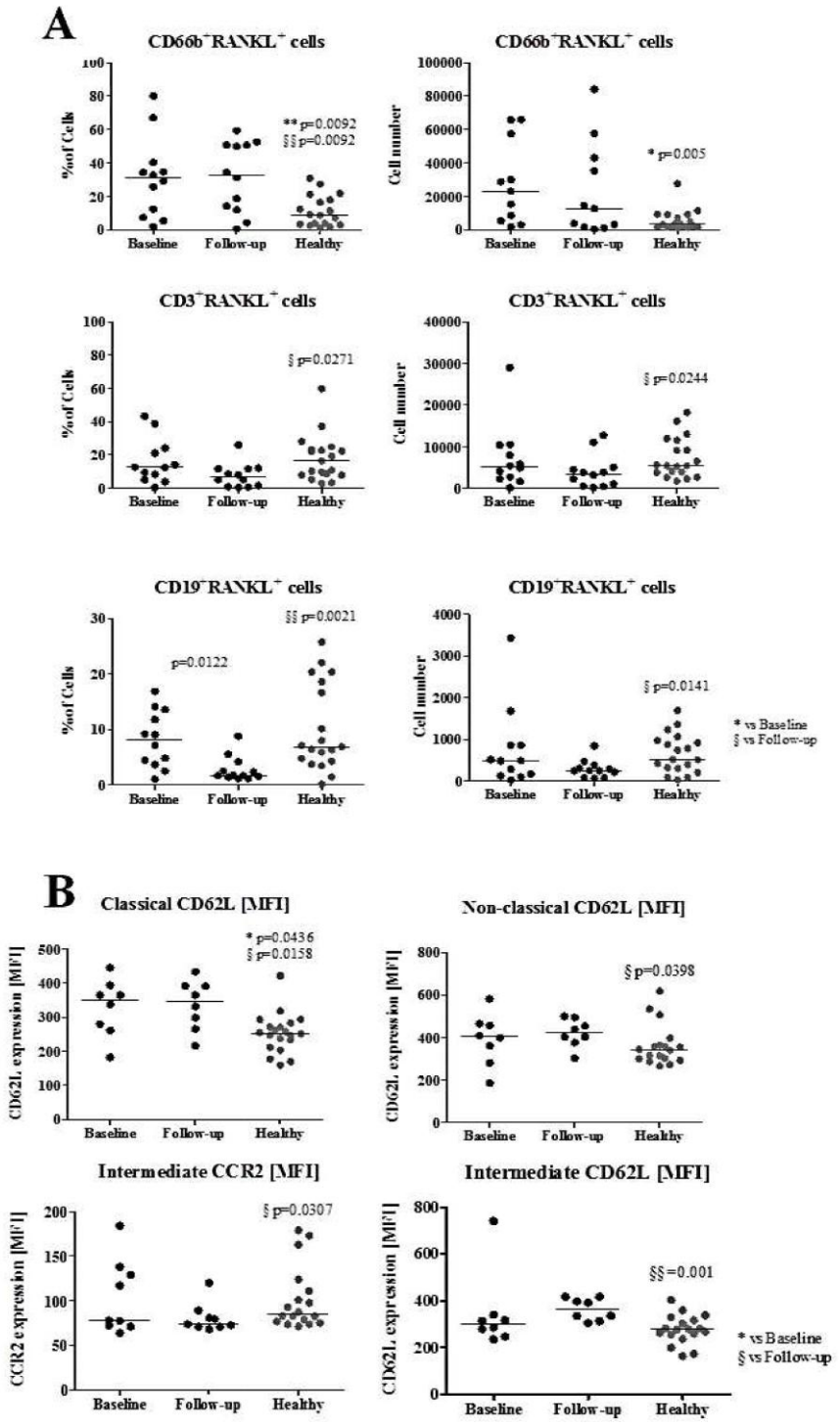

Figure 1. *RANKL surface expression in leukocytes and monocyte phenotype* A) $RANKL^+$ frequency and absolute number in circulating leukocytes. $CD66b^+RANKL^+$ cells are increased in the circulation of patients at baseline and at follow-up when compared to healthy donors (frequency p=0.0092; absolute number p=0.005). At follow-up, $CD3^+RANKL^+$ are decreased in circulation when compared to healthy donors (frequency p=0.0271; absolute number p=0.0244). $CD19^+RANKL^+$ frequency is decreased after treatment when compared to patients at baseline (p=0.0122) and with healthy donors (p=0.0021). This difference is also observed at the absolute number level when compared to healthy donors (p=0.0141). $RANKL^+$ cells were analysed by flow cytometry and gated inside each subpopulation. B) Phenotype of circulating monocyte subpopulations - Classical $CD14^{bright}CD16^-$, intermediate $CD14^{bright}CD16^+$, non-classical $CD14^{dim}CD16^+$. CD62L is increased in the circulating

classical subpopulation of patients at baseline (p=0.0436) and at 6 months follow-up (p=0.0158) when compared to healthy donors. CD62L expression is increased in patients after 6 months follow-up both in the non-classical subpopulation (compared to healthy p=0.0398) and in the intermediate subpopulation (compared to healthy p=0.001). CCR2 expression is reduced in the intermediate subpopulation of patients at follow-up when compared to healthy donors (p=0.0307). Each dot represents a sample. Line represents median. * vs Baseline, § vs Follow-up. * and § p<0.05, ** and §§ p<0.01. MFI - Median fluorescence intensity.

IL-17A, IL-23 and TGF- β circulating levels are reduced in AS patients after TNFi treatment

DKK-1, IL-1β, IL-6, IL-17A, IL-12p70, IL-23, TNF, MCP-1 and TGF-β levels were significantly higher in patients at baseline when compared to healthy donors (Fig.2). After correcting for multiple comparisons only IL-1β, IL-23, MCP-1 and TNF remained significantly higher in patients at baseline when compared to healthy donors

Circulating levels of IL-17A, IL-23 and TGF-β were decreased after TNFi treatment when compared to baseline (p=0.0195, p=0.0098 and p=0.0115, respectively; Fig.2). CTX-I levels were lower in patients at 6 months of follow-up when compared to healthy donors (p=0.0268; Fig.2). After correcting for multiple comparisons, none of the markers were significantly decreased after treatment.

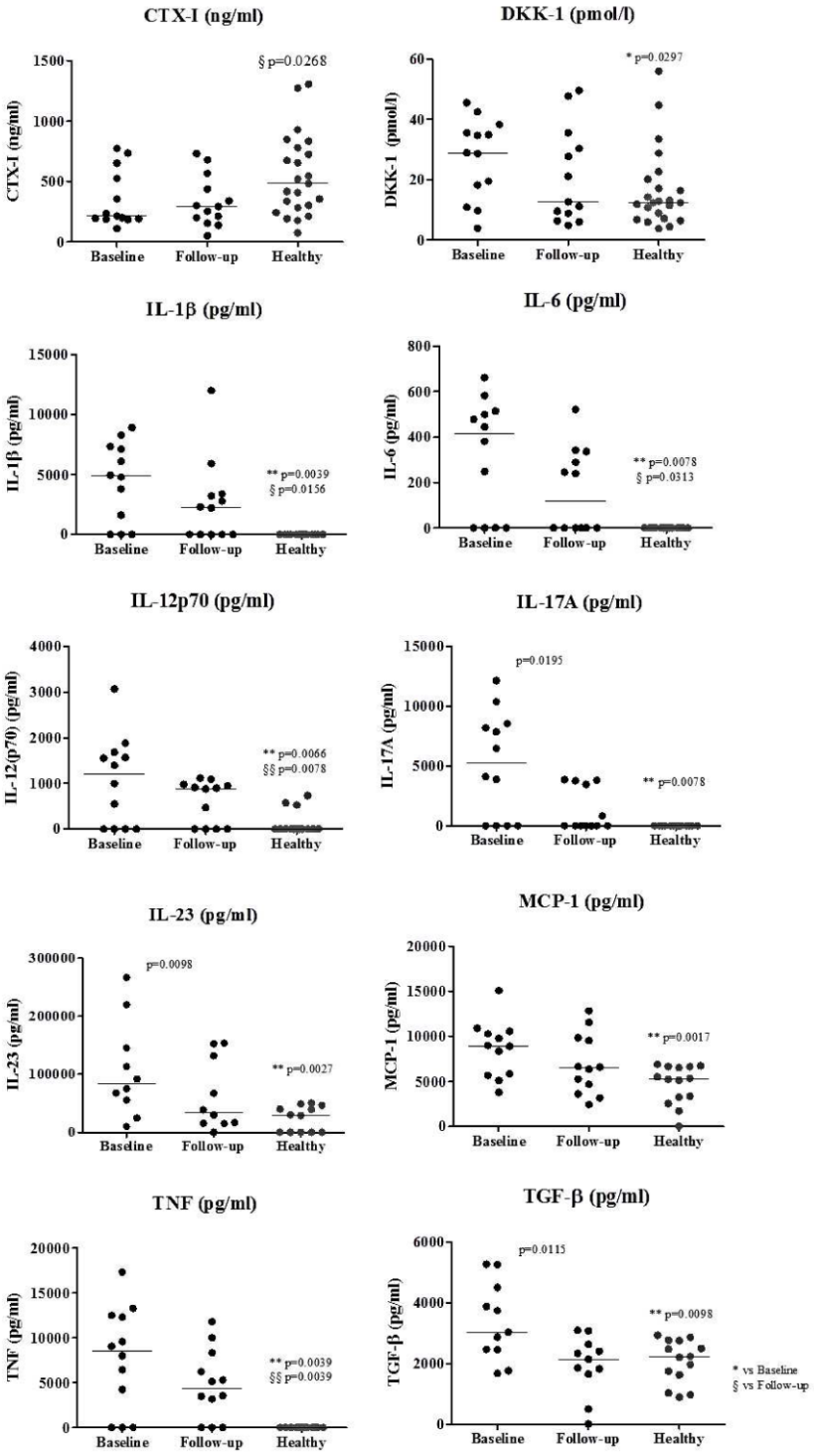

Figure 2. *Serum levels of bone turnover markers, bone metabolism proteins and cytokines.* CTX-I levels are decreased in patients at 6 months follow-up when compared to healthy donors (p=0.0268). DKK-1, IL-1 β, IL-6, IL-17A, IL-12p70, IL-23, TNF, MCP-1 and TGF-β are increased in patients at baseline when compared to healthy donors. After 6 months of therapy, follow-up patients had decreased levels of IL-17A, IL-23 and TGF-β when compared to their baseline. We have also observed that after therapy the levels of IL-1 β, IL-6, IL-12p70 and TNF were still significantly higher than healthy donors levels'. Each dot represents a sample. Line represents median. * vs Baseline, § vs Follow-up. DKK - dickkopf-related protein, CTX - carboxy-terminal collagen crosslinks, IL - interleukin, TNF - tumor necrosis factor, MCP - monocyte chemmotractant protein, TGF - transforming growth factor.

Osteoclast differentiation from circulating precursors in AS patients is lower than in healthy controls and osteoclast-mediated bone resorption is increased after TNFi treatment

Under stimulation, reproducing local bone inflammatory microenvironment, AS patients, prior to TNFi, had less OC differentiation at culture day 21 than healthy donors (p=0.0038; Fig.3A). However, the number of OC increased throughout the culture period in all the studied groups. No differences were found in the number of nuclei per osteoclast between the studied groups. Both resorption pit number and percentage of resorption area were markedly increased after culture day 14 in cells from patients treated with TNFi as compared to baseline, reaching statistical significance at culture day 21 (p=0.0469 for both resorption pit number and percentage of resorbed area; Fig.3B).

Gene expression by RT-qPCR was performed for OC genes that are known to be important during its differentiation and activity. All genes, except CTSK, were significantly lower at culture day 1 in patients after TNFi treatment when compared to healthy donors (CSF1R p=0.0186; RANK p=0.0095; NFATc1 p=0.0015; ATP6V0D2 p=0.0004; Fig.4). At culture day 1 RANK expression in patients at baseline was significantly lower than in healthy donors (p=0.0268; Fig.4). There were no differences between patients and controls or between baseline and post TNFi treatment patients, except for ATP6V0D2 expression at culture day 21 in patients at baseline, which was significantly lower than in healthy donors (p=0.039; Fig.4) and than in patients after TNFi (p=0.0234). After correcting for multiple comparisons, expression of RANK, NFATc1 and ATP6V0D2, in culture day 1, from patients after TNFi remained significantly lower than in healthy donors.

No differences were found in any of the studied parameters when comparing presence or absence of HLA-B27, presence or absence of peripheral involvement or comparing monoclonal antibodies (Adalimumab, Infliximab or Golimumab) to the fusion protein Etanercept (data not shown).

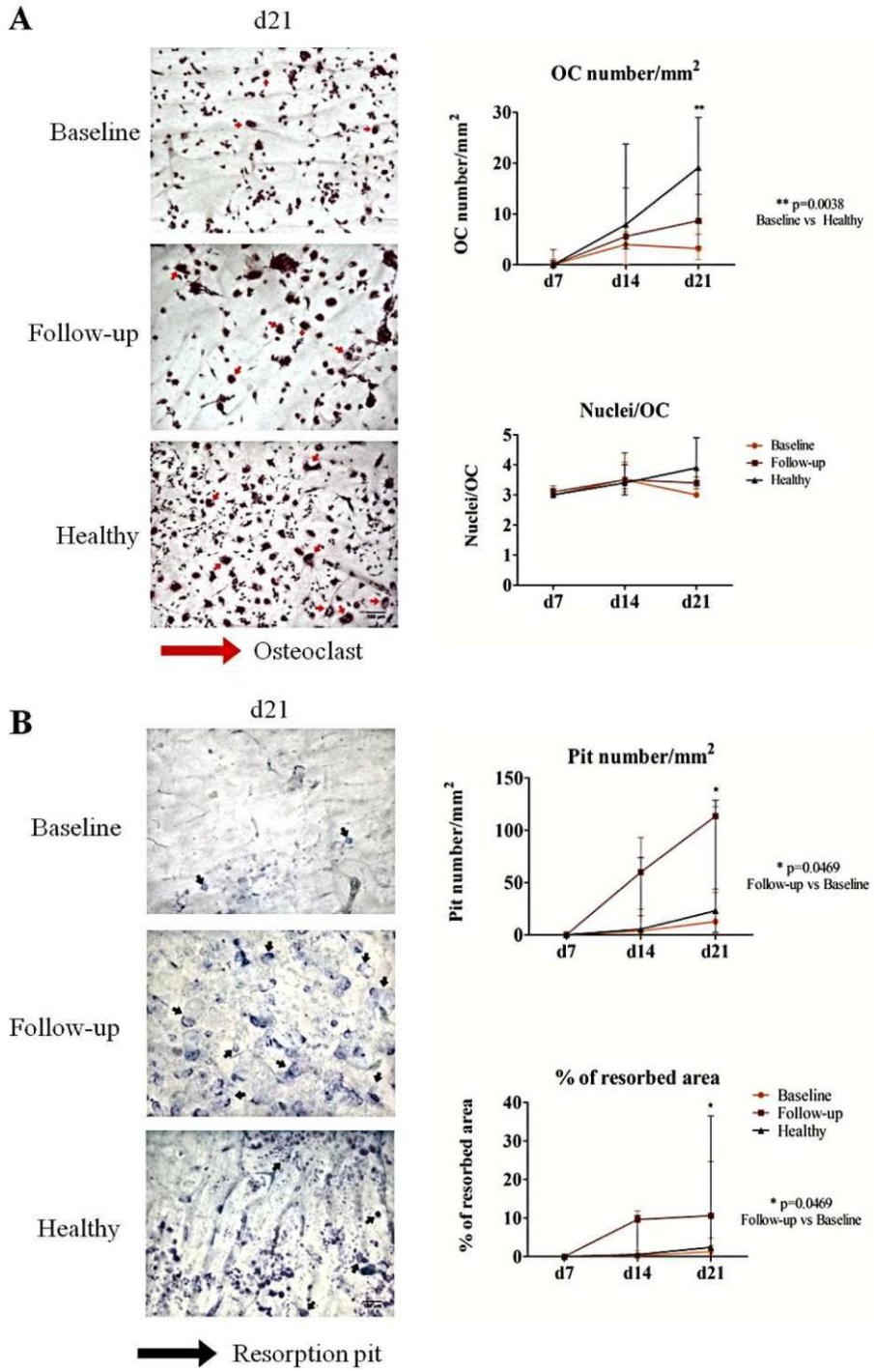

Figure 3. *Osteoclast number is reduced in baseline patients, but bone resorption activity is increased after TNF-blocker exposure.* A) Representative images of culture day 21 of cells stimulated with M-CSF, RANKL, dexamethasone and TGF-β and stained for TRAP. OC number increased throughout time and at culture day 21 baseline patients have significantly less osteoclasts than healthy donors (p=0.0038). No differences were found in the number of nuclei per OC in any studied time of culture. B) Representative images of pit assay at culture day 21. Patients at follow-up had significantly higher number of pits and resorption area at culture day 21 when compared to their baseline (p=0.0469 for both resorption pit number and percentage of resorbed area). Dots represent median counts for each group at each time-point and bars represent interquartile range [10-90]. d - day; OC - osteoclast; Scale bars 100µm, red arrows - osteoclasts, black arrows - resorption pits.

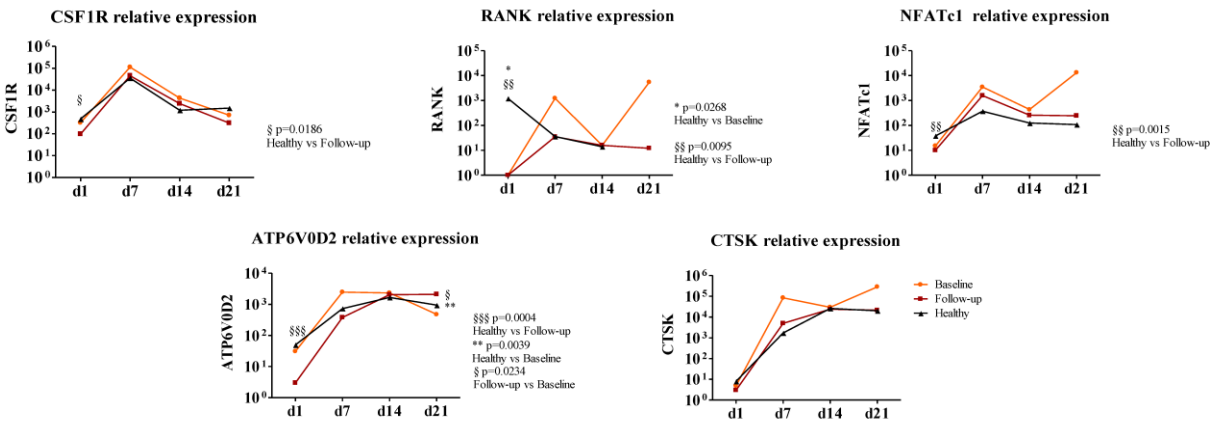

Figure 4. *Gene expression profile of stimulated cells in culture for 21 days.* All genes except CTSK are significantly decreased at day 1 in patients at follow-up when compared to healthy donors. At day 1 RANK expression in patients at baseline is also significantly reduced when compared to healthy donors (p=0.0268). We found no differences between groups throughout the culture time except for Atp6v0d2 expression at day 21 in patients at baseline which is significantly reduced when compared both to healthy donors (p=0.039) and patients at follow-up (p=0.0234). Relative gene expression shown in Log scale. Dots in graphs represent median gene expression for each group at each time-point. d - day; CSF1R - gene encoding macrophage-colony stimulating factor (c-fms), RANK - gene encoding for receptor activator of nuclear factor-κβ, NFATc1 - gene encoding for nuclear factor of activated T-cells, Atp6v0d2 - gene encoding ATPase, H^+ transporting, lysosomal V0 subunit D2, CTSK - gene encoding cathepsin K. $p<0.05$ is considered significant.

Discussion

We have shown that in TNFi treated AS patients, the pro-inflammatory and pro-osteoclastogenic systemic stimuli were decreased due to reduced $RANKL^+$ circulating lymphocytes (B and T cells) and reduced levels of IL-17A and IL-23. Accordingly, OC specific gene expression was reduced in circulating precursors after TNFi exposure. However, when these precursors from TNFi treated AS patients were cultured in OC differentiating conditions, reproducing the bone microenvironment, their response to osteoclastogenic stimuli and activity was increased in comparison to baseline behavior.

One of the limitations of our study is the sample size and these results should be confirmed in a larger population. Another limitation is the use of circulating precursors. Bone local samples would have been preferred to use, however surgery to this bone areas of interest are rare and in addition it would be most difficult to obtain healthy controls for these samples. Our

strategy was to address the question of circulating precursors being an important source of osteoclasts in active disease and the fate of their osteoclast differentiation ability after exposure to TNFi.

Our study found no correlation between monocyte phenotype, osteoclast activity or gene expression and treatment duration. Although there have been suggestions that longer therapy duration increases bone mineral density in AS patients [20] these studies have been performed in longer time courses than our study. We also found no differences between the use of monoclonal antibodies (Adalimumab, Golimumab and Infliximab) and the recombinant protein Etanercept. Most of the literature does not compare different TNFi [21, 22] and thus more studies are needed to address if differences might exist at the level of radiographic progression.

Recent studies and meta-analysis have shown that TNFi treatment in AS patients is associated with increased lumbar and hip BMD [23]. In addition, an increased likelihood of developing new bone following resolution of inflammation after TNFi therapy has been suggested. Accordingly, radiographic progression was associated with decreased systemic inflammation and, on the contrary, radiographic nonprogression was associated with persistent inflammation, as assessed by IL-6 and CRP levels and MRI, supporting a link between the resolution of inflammation and new bone formation in AS patients during TNFi therapy [24, 25].

It has been previously reported that neutrophils are more active in AS patients [26] but no differences in total B or T cell numbers were reported [27]. In our study no differences were found in the frequency of granulocytes and T and B cells between any of the studied groups. However, $RANKL^+$ neutrophils count was increased in patients at baseline and TNFi treatment reduced the number of $RANKL^+$ T and B cells. Previous studies have shown that T lymphocytes from AS patients have higher expression of RANKL than healthy donors [28],

but to our knowledge a comparative study of RANKL expression in AS patients before and after TNFi was never been published.

In previous studies AS patients under NSAID therapy have been showed to have increased circulating number of classical monocytes and decreased non-classical monocytes when compared to healthy donors [29]. However, in our cohort, no differences were detected in any of the circulating monocytes subpopulations. In the intermediate monocyte subpopulation, patients exposed to TNFi had decreased CCR2 expression. CCR2 is a chemokine receptor that binds MCP-1 and promotes osteoclast precursors fusion and maturation [30]; its reduction after treatment is in accordance with the reduced gene expression of specific osteoclast genes observed in cells from patients after TNFi treatment and with its previously described role in osteoclastogenesis [30, 31]. On the other hand, CD62L (L-selectin) was higher in AS patients after TNFi therapy in all three monocyte subpopulations when compared to healthy donors suggesting that adhesion is increased in these cells after exposure to TNFi. We speculate that our observation of high L-selectin (CD62L) expression in circulating monocytes subpopulations after TNFi treatment may be related to increased adhesion of OC precursors to bone slices and subsequent cell activation. It was previously described in rats that the binding of L-selectin to some of its ligands (namely GlyCAM-1) increases integrin binding (β2 and also α4) [32-34]. Therefore, binding of L-selectin to ligands on the bone slice might increase αvβ3 integrin binding leading to increased OC differentiation [35]. We suggest that when osteoclast precursors attach to bone slices through integrins and L-selectin (CD62L), signaling pathways are activated and the expression of OC differentiation genes is induced. It has been previously shown that attenuation of the integrin $\alpha_V\beta_3$ pathway leads to inhibition of OC differentiation and that there is a crosstalk between integrin β_3 and M-CSF/c-fms pathways [36]. We further suggest that cell adhesion to bone by

integrins plays an important role in OC differentiation, but additional studies are required to determinate how integrins are able, *per se*, of inducing OC differentiation.

As previously reported, serum levels of IL-1β, IL-6, IL-12p70, IL-17A, IL-23, MCP-1, TGF-β and TNF were significantly higher in AS patients with active disease when compared to healthy subjects [37, 38]. In addition, IL-17A, IL-23 and TGF-β were significantly decreased in AS patients after TNFi therapy. IL-17A and IL-23 are well known for their role in the pathogenesis of inflammatory disorders, such as AS [39], and TGF-β is an important cytokine for bone formation [40]. In accordance with our study, Limón-Camacho *et al* found that serum levels of IL-17A were significantly elevated in AS patients with active disease, when compared to patients receiving TNFi; the same findings were observed for IL-6, IL-12, and TNF [37]. Moreover, we observed that the levels of most cytokines present in the serum of AS patients normalized to the healthy donors levels after 6 months of TNFi therapy, indicating a reduction in the inflammatory environment induced by TNF blockade. Concordantly, patients under TNFi had reduced levels of CTX-I, suggesting a decrease in osteoclast activity. However, no differences in the levels of sRANKL, OPG and their ratio, before and after TNFi were found. Previous studies have shown discrepancies in sRANKL and OPG levels in AS patients. While sRANKL and OPG have been found increased in AS patients with active disease [41], in patients with mild to active disease sRANKL/OPG was lower than in healthy controls [38, 42]. These latter studies found that after TNFi, sRANKL/OPG ratio was increased due to a decrease in OPG; however, none of the studies used paired patients samples.

SOST and DKK-1 have been implicated in the pathophysiology of AS, either by their reduced expression or by their functional de-regulation [43]. In our study, DKK-1 serum levels were higher in patients at baseline when compared to healthy controls; however, no significant differences in SOST or DKK-1 serum levels after treatment were found. Taylan *et al* reported

that patients under TNFi presented higher DKK-1 levels compared to patients under conventional therapy (NSAIDs and/or DMARDs) [38]. More recently, Ustun *et al* found that TNFi did not affect DKK-1 and SOST levels [44]. However, several other studies suggested that DKK-1 levels decrease after TNFi and that there is no change in SOST circulating levels [43].

To understand the effect of TNFi in osteoclast differentiation and function we isolated PBMCs from AS patients before and after TNFi and cultured them *in vitro* over bone slices. OC formation continuously increased up to day 21. Both controls and patients (before and after TNFi treatment) exhibited the same pattern of increase in the number of resorption pits and the percentage of resorbed area over time, although at day 14 there was a marked increase in resorption in the TNFi treated patients that reached statistical significance at day 21. After TNFi treatment no differences in pit size were found suggesting that OC mobility is not affected by the disease or therapy and we found no differences in the number of nuclei/osteoclast, which has been suggested to correlate with osteoclast activity [45-47].

There is evidence that TNF contributes to expression of specific OC proteins and that it directly activates OC differentiation through cross activation of the NF-κB pathway or c-Jun N-terminal kinase (JNK) signaling cascade [48]. In our study, all genes, with the exception of cathepsin K, were downregulated after TNFi treatment. However, this reduced gene expression did not impair OC differentiation when the PBMCs were cultured under OC differentiating conditions. At later time points, culture days 14 and 21, there was a significant increase in ATP6V0D2 expression in healthy and TNFi treated patients, which might be related to our observation of increased bone resorption after TNFi.

In summary, in AS patients TNFi treatment reduces systemic pro osteoclastogenic stimuli. However, TNFi effect on OC precursors from AS patients increases their response to osteoclastogenic stimuli and their activity in bone pro inflammatory microenvironment. This

is in disagreement with the apparent increase in osteoproliferation in AS patients treated with TNFi [24, 25]. However, patients treated early in the course of the disease appear to escape this effect [49]. We hypothesize that early TNFi treated patients have an early normalization of bone resorption by TNFi, thus preventing osteoproliferation.

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Discussion

RA is a highly destructive disease characterized by inflammation of the joints, hyperproliferation of the synovial membrane, with consequent cartilage and bone destruction. Bone erosions and secondary osteoporosis are a downstream effect of inflammation and of the partial dissociation between bone resorption and bone formation. Local activation of OC precursors, together with the production of MMPs, leads to cartilage and bone destruction and erosion. Bone formation is impaired by an increase in Wnt pathway inhibitors like DKK1 and SOST, however it is not clear if these are the only mechanisms blocking bone formation [275, 536, 537]. In contrast to RA, in AS joint and spine, damage is characterized by new cartilage and bone formation leading to ankylosis of the sacroiliac joints and spine. Although the affected sites are anatomically different, systemic and local inflammation are present in both diseases. While RA is essentially associated with erosive joint bone lesions, AS induces osteoproliferation at specific sites of the skeleton, pointing towards the existence of subtle differences between RA and AS in the regulation of osteoclastogenesis. Due to excessive proliferation without being compensated by resorption, we have hypothesized that OC from AS patients have an impairment either in differentiation or activity.

The main goal of the present work was to study the differentiation and activity of circulating OC precursors from RA and AS patients with active disease and the effect of conventional DMARDs and TNFi therapy in these cells.

We first addressed the cytokine and chemokine environment in early RA patients. To this purpose, a cohort of untreated polyarthritis patients with less than six weeks of disease duration was followed up, allowing the identification of a subset of patients that later on evolved to RA, defined as very early RA (VERA) patients. The remaining patients either had self-limited forms of arthritis or evolved into other chronic inflammatory joint diseases, and were classified as very early arthritis (VEA) patients. These patients were evaluated at baseline without therapy, after short-term treatment with low-dose corticosteroids and after 4 months of MTX.

We found that VERA patients have a cytokine profile that supports neutrophil recruitment (IL-8) and Th17 cells polarization (IL-1β and IL-6) and activation (IL-17A and IL-22) when compared with VEA and established RA patients under MTX treatment. These cytokines are also involved in the activation of monocytes and of the inflammatory cascade, as well as in OC activation [253, 538]. The analysis of synovial fluid samples from established RA patients revealed a similar cytokine pattern. Considering the cytokine profile observed in the very early phase of the disease, we propose that after an initial

unknown starting event where autoantibody production is triggered, macrophages and neutrophils migrate to the joints and are activated by MCP-1 and IL-8 chemotactic gradients, respectively [539, 540]. In fact, previous results demonstrated that these cells heavily infiltrate the synovial tissue during disease onset [541]. Hence, neutrophils and macrophages are the most abundant cells infiltrated into the joints of RA patients and the latter can be triggered to differentiate to OC [253]. In the synovial tissue, the production of immune complexes that deposit in joints and activate Fcγ-receptors expressed by macrophages [542-544], further increases the inflammatory process through the production of pro-inflammatory cytokines, such as TNF and IL-1β by macrophages [455, 542, 545]. In addition, IL-1β can stimulate endothelial cells, T and B cells, and fibroblasts to produce RANKL, IL-6 and IL-8. Furthermore, IL-1β, together with IL-6, is able to drive Th17 cells polarization [546, 547]. Activated Th17 cells produce high levels of IL-17A, which is able to extend neutrophil survival [548, 549] and stimulate osteoclastogenesis leading to bone destruction [341, 343, 550]. Moreover, increased IL-6 levels support a continuous recruitment of autoreactive B cells toward the synovium during RA progression, contributing to the chronic inflammatory process [551, 552].

It has been reported that in patients before disease onset, there are increased levels of several circulating cytokines (including IL-1β, IL-6, IL-12, IL-17 and TNF) associated with the development of inflammation and possibly predictive of the onset of RA. The same report shows that IL-17 concentration was present at its highest level in prearthritis patients and decreased afterwards in RA patients after more than 6 months of disease duration [553]. This is in accordance with our observations that the concentration of IL-17A was increased in VERA patients but similar to healthy controls in the established phase of the disease. Interestingly, it was also demonstrated that in DMARD-naïve RA patients with less than 2 years of disease duration there are increased circulating levels of Th1 and monocyte/macrophage-derived cytokines, possibly counter-regulated by cytokines produced by Th2 cells. In contrast, IL-17 was barely detectable in this cohort of patients, which is in agreement with the idea that the early phase of RA is influenced by a Th17 profile, which is gradually diluted during the progression of the disease [553, 554]. IL-17 and Th17 were recently regarded as target therapies for RA. Indeed several anti-IL-17A (ixekizumab and secukinumab) and IL-17RA (brodalumab) monoclonal antibodies have been used in randomized controlled trials for RA patients. These trials showed less promising results than expected [555]. Moreover, there are subsets of patients that do not respond to the therapy and it is proposed that this is due to the change of Th17 to Th1

profile during disease progression or due to an heterogeneity of IL-17 (A and F) and its receptors' (IL-17RA and IL-17RC) expression in the synovium of patients [556]. Our results suggest that IL-1β, IL-6 and IL-17A are the cytokines most likely to be relevant in the first few weeks of RA, where the transition to the chronic phase of the inflammatory process occurs.

Currently, the gold standard treatment for RA at the time of presentation is MTX. Interestingly, neither the initial therapy with low dose corticosteroids nor MTX affected cytokine production in VERA patients. Thus, despite being able to reduce clinical manifestations of the disease, these conventional therapies were ineffective in reversing the proinflammatory cytokine production that underlies inflammation in VERA patients, leaving an uncontrolled sustained stimulus on osteoclastogenesis in early RA patients.

We next aimed to investigate specifically the OC precursor behaviour in patients treated with MTX and we recruited a cohort of early RA patients (less than one year of disease evolution). These patients were evaluated at baseline and after 6 months of MTX therapy. We observed that MTX treatment only decreased the number of circulating neutrophils having no effect on the frequency of T or B lymphocytes. No effects were observed in the surface expression of RANKL or in serum levels of sRANK and OPG in these patients. Although MTX therapy did not affect the frequency of monocyte subpopulations it decreased the expression of activation and adhesion markers, but importantly decreased the surface RANK expression in the intermediate and non-classical subpopulations. According to our aim, we then studied OC differentiation from these patients' circulating precursors. In RA patients exposed to MTX we did not find any significant differences in OC numbers after treatment and only a small decrease in the resorbed area per cell was noted.

It has been previously suggested that MTX and biologic drugs target distinct pathways of the disease [557]. MTX stimulates adenosine release, suppressing the inflammatory function of neutrophils, macrophage/monocytes, dendritic cells and lymphocytes through a decrease of IL-12, TNF, MIP-1a and nitric oxide production, while increasing IL-10 [558-560]. High dose MTX used in cancer therapy has been associated with bone loss through a negative influence on osteoblasts; however, in low dose (around 10 mg/week) MTX use has no deleterious effects in bone metabolism [561, 562]. It has been shown that MTX reduces the expression of RANKL in synovial fibroblast cultures [367, 563] and more recently it was associated with a decrease in RANKL/OPG ratio and reduction of DKK1 levels [368, 564].

We then studied the effect of TNFi in RA patients circulating OC precursors by recruiting a cohort of patients and analyzing it before and after TNFi exposure.

TNFi decreased the surface RANKL expression on B lymphocytes and we also showed that the sRANKL/OPG ratio was diminished in TNFi treated patients due to a reduction in serum RANKL levels. TNFi declined the frequency of the classical subpopulation, defined as the OC precursors [565], and increased the non-classical subpopulation. In the TNFi treated patients RANK expression was lower after treatment but not to a significant extent. In line with these observations we found that in RA patients under TNFi not only the number of OC was decreased, but also the resorbed area was diminished. This effect was associated with an initial downregulation of TRAF6 and Fra2 expression and a later downregulation in cathepsin K expression.

TNFi have shown efficacy in improving clinical, functional and radiographic outcomes, especially if administrated early in disease course [369, 388] and generally the effect is optimized when in combination with MTX [384, 566-571]. TNFi downregulates pro-inflammatory cytokines, rheumatoid factor and ACPA levels and reduce leukocyte trafficking, angiogenesis and consequently joint destruction [572-576]. These drugs also decline $CD4^+$ T cell activation both in the blood and in the synovial fluid and upregulate Treg suppressive function [577, 578]. TNF blocking therapies arrest radiographic damage in RA [374, 389-391]. Consistent with that the increase in the number of OC precursors in RA patients' PBMCs compared to normal controls appears to be reversed with TNFi therapies [394]. TNFi improve RA outcome not only by blocking local and systemic inflammatory cascades but also by providing beneficial effects against joint and bone damage [566, 567]. Indeed, TNFi have a positive impact on the secondary systemic osteoporosis associated with RA. For instance, both infliximab and etanercept were shown to increase BMD in the spine and hip after 6 months and up to 1 year follow-up [579, 580] [566] or at least were able to halt bone loss [390]. Focal bone loss, such as hand bone loss, is more influenced by local disease activity and might be less influenced by TNFi if local activity persists. In the BeSt study hand BMD decreased despite increased lumbar and hip BMD after 1 year infliximab [581], indicating that if local joint inflammation remains active it will lead to localized bone loss and structural joint damage, despite low global disease activity [582].

Our next step was to study the circulating OC precursors in AS patients and for this purpose we recruited a cohort of untreated AS patients where we observed an increase in

many of the pro-osteoclastogenic cytokines, such as IL-1β, IL-6 and TNF, but not of IL-17 or IL-23, when compared to controls. We also found less surface RANKL expression in neutrophils, despite increased numbers of RANKL expressing neutrophils. Regarding OC circulating precursors we found a decrease in the non-classical subpopulation in AS patients when compared to controls and the major differences we observed between the two groups were in the phenotype of monocytes. In AS only CD62L (the L-selectin) and the OC specific markers (CD51/CD61 and RANK) were altered when compared to controls. These markers were increased in the non-classical and intermediate subpopulations of monocytes of AS patients when compared to controls. However the vitronectin receptor (CD51/CD61, $\alpha_v\beta_3$ integrin) expression was reduced in the classical subpopulation of AS patients, suggesting that some of the OC circulating precursors might be unable to attach to bone matrix and differentiate to OC. *In vitro* culture showed no differences between AS patients and healthy donors in the number of differentiated OC or in their activity, despite originating from a highly pro-inflammatory environment. Since AS patients have increased serum levels of pro-osteoclastogenic cytokines, but AS circulating OC precursors and differentiated OC showed no differences when compared to healthy donors, we performed gene expression in cells differentiated from AS OC precursors and found that on the first day of culture, without any stimuli, the expression of OC key genes like RANK, CSF1R (M-CSF receptor) and NFATc1 (an important OC transcription factor) was downregulated. This was also true on the last culture day where both CSF1R and NFATc1 were still decreased, as well as the genes that code for the subunit of H^+Atpase and cathepsin K, both important in OC resorption activity.

Several evidences point to disturbed monocytes subpopulations in AS patients but the literature is not consistent with regards to which subpopulation is most affected. While some studies support our findings of a decrease in the non-classical subpopulation in AS [583], other studies have reported that axial spondyloarthritis patients (including AS patients) had increased circulating number of classical monocytes when compared to healthy donors, but found no differences in monocyte surface markers [584].

This result in the peripheral blood is also of particular interest when we speculate what is happening in AS patient's joints and enthesis since it was previously shown that immune system cells are present locally and express pro-inflammatory cytokines [434, 449, 450, 460, 461]. Studies on both zygapophyseal and sacroiliac joints showed that AS patients had increased number of OC and less OB than healthy donors [449, 461, 464, 465, 467]. These studies are limited to sample availability, constraints on disease duration and lack of

appropriate controls, so there is a need for further studies in AS patients in the areas of new bone formation to understand if the decreased OC precursor response that we find in circulating precursors is also present locally in bone.

We then analysed the effect of TNFi therapy in the circulating precursors of AS patients. TNF blockade is highly effective in targeting the different disease features – not only axial disease but also peripheral arthritis, enthesitis and extra-articular features, such as psoriasis or uveitis, and on general symptoms such as fatigue and substantially improves the overall function and quality of life [500-503]. It seems that patients early in the course of their disease, with high CRP concentrations, positive MRI findings and less structural damage are more likely to respond positively than patients with advanced disease, although all subgroups benefit from this treatment [505]. Both RA and AS share common inflammatory pathways that can be blocked by targeted therapies like TNF inhibitors, so we asked the question of what would happen when circulating OC precursors from AS patients are exposed to TNFi therapy. Would they be further inhibited?

To understand the behaviour of circulating osteoclast precursors in TNFi treated AS patients we followed-up a cohort of AS patients before and after TNFi therapy. We found that TNFi did not affect circulating leukocyte subpopulations besides a decrease in $RANKL^+$ B cells; however, TNFi treated patients had lower circulating levels of IL-17 and IL-23 when compared to baseline, reaching normal levels. Despite the similar findings in RA, TNFi in AS patients had no effect in the circulating sRANKL/OPG levels, nor in monocytes' frequency or phenotype. We then characterized the circulating osteoclast precursors' ability to differentiate *in vitro* and observed that although the number of OC remained the same, after TNFi they showed increased ability to resorb bone than baseline patients or healthy donors. Moreover, cells from TNFi treated patients exhibited higher expression of H^+Atpase, which we speculate is responsible for increased bone resorption.

The direct effect of TNFi in cell differentiation has not been extensively studied and there are contradictory results. An earlier study showed that infliximab directly enhanced RANKL-dependent osteoclastogenesis and OC activity from PBMCs [585] and more recently it was shown that direct treatment of PBMCs with infliximab decreased OC forming ability and activity [586]. These results are not in line with the study of Gegenbacher that found that OC from AS patients' circulating precursors under TNF blockers had decreased bone resorption activity than at baseline. In this study PBMC were seeded in alpha-MEM media and supplemented with 1,25-dihydroxycholecalciferol, dexamethasone, M-CSF and RANKL and differentiated to OC and OC activity was

measured solely by pit number [390]. Although not extensively studied, the use of 1,25-dihydroxycholecalciferol or 1,25-dihydroxyvitamin D3 in OC culture is not consensual, since it was shown that osteoclastogenesis was inhibited in the presence of this molecule [587, 588]. These differences in the protocol can partially explain the differences between our own study and the study by Gegenbacher.

We determined that cells from AS patients have impaired OC gene expression and we propose that this happens as a result of disease specific mechanisms, such as epigenetic regulation.

Previous studies found a specific profile of reduced activity of histone deacetylases and histone acetyltransferase in AS patients and that specific sites of DNA methylation were associated with disease activity and cytokine production [589, 590]. MicroRNAs (miRNAs, miRs) are a group of endogenous, 20–25 nucleotides long non-coding RNAs that bind to RNA and inhibit transcription of target genes [591, 592]. Overexpression of microRNA (miR)-16, miR-221 and let-7i was also found in AS T lymphocytes and both miR-221 and let-7i were positively correlated with radiographic changes in lumbar spine [593, 594]. Recent studies have also tried to find alterations in the microRNA profile of AS patients after TNFi therapy but no associations with clinical parameters were found [595]. OC differentiation is also subject to epigenetic regulation. RANKL induced changes in histone modification stabilize NFATc1 expression but there are other mechanisms also triggered by RANKL and also M-CSF that participate in the negative feedback loop of NFATc1 expression. Interestingly several microRNAs have been also involved in the regulation of osteoclastogenesis like miR-223, miR-21, miR-124 and miR-155 [292, 596-600]. MiR-223, miR-21 and miR-155 have been implicated in RA pathogenesis [443, 601, 602] and miR-21 and miR-124 have been implicated in AS [603, 604]. MiR-21 increased expression in whole blood of AS patients was associated with increase in c-fos expression, OC formation and in serum levels of CTX-I [599, 604]. MiR-124 is downregulated by RANKL and targets NFATc1 expression [605]. It was previously shown that miR-124 decreases osteoclastogenesis and inflammation in a mouse model of arthritis [606]. Moreover, in animal models it was also shown to target STAT3 and reduce IL-6 and ROS production, and to target TACE impairing TNF release [606-608]. Recently, Xia and colleagues showed that miR-124 expression was increased in whole blood of AS patients and was associated with decreased anthrax toxin receptor 2 (ANTXR2) expression [603]. This led to increased JNK phosphorilation and the authors associated it with autophagy related cell death in the gut of AS patients and increased expression of IL-23 [603, 609,

610]. ANTXR2 has functions in basement membrane matrix assembly, angiogenesis and embryonic development and is associated with AS by genome-wide association studies, however its role in AS pathogenesis is still unknown [611-614]. Further studies are needed to understand the role of miR-124 in monocytes in AS and in their ability to differentiate to OC and in the effects of TNFi therapy specifically on this miR in AS context.

RA and AS are two very distinct diseases in some aspects, such as the predominantly affected gender, age of onset, anatomical involvement, disease activity assessment, functional impact, structural damage and treatment strategy. This makes direct comparisons between groups of RA and AS patients very difficult. The results of our separate evaluations of RA and AS patients indeed suggested that OC from AS patients have an impairment either in differentiation or activity. These very interesting results motivated us, even taking into consideration the methodological limitations of this exercise, to analyse and discuss, in an integrated approach, our observations in AS and RA patients. In table 3 we have summarized our experimental findings from results chapters II and IV, where we compared RA and AS with their respective control groups.

We found a lower number of non-classical monocytes in AS patients when compared to controls, which was not observed in RA patients. Several evidences point to disturbed monocytes subpopulations in RA and AS patients but the literature is not consistent regarding which subpopulation is most affected. While some studies support our findings of a decrease in the non-classical subpopulation in AS patients [583], other studies in RA and AS have reported contradictory findings to ours, describing that RA patients had increased numbers of circulating intermediate monocytes when compared to controls [349] and that AS patients had increased circulating classical monocytes, when compared to healthy donors [584]. It has been postulated that the classical monocyte subpopulation would be the typical OC precursor subpopulation. However, it has already been shown that all monocyte subpopulations can differentiate to OC [226, 246, 615].

Table 4 summarizes our findings when we compared RA and AS patients directly and after statistical correction for symptom duration.

The major differences we observed between the two diseases were in the monocytes phenotype. While in RA we found increased expression of classical activation markers, adhesion molecules and OC specific receptors, consistent with previously published studies [616]; in AS only CD62L (the L-selectin) and the OC specific markers (CD51/CD61 and RANK) were altered when compared to controls. When we compared

the two groups of patients we found that in all monocyte subpopulations several surface markers of activation were increased in RA, when compared to AS, but after correcting for symptom duration only classical CCR2 and CD86 and non-classical CD51/CD61 remained significantly increased in RA.

Table 3 - Summary of the findings of RA and AS patients when compared to age and gender matched healthy controls.

Parameter	RA vs Healthy	AS vs Healthy
Leukocyte frequency*		
Neutrophils	↑	↑
T cells	=	=
B cells	=	=
RANKL expression*,#		
Neutrophils	=	↓
T cells	=	=
B cells	=	=
Monocyte subpopulation frequency*		
Classical	=	=
Intermediate	=	=
Non-classical	=	↓
Monocyte phenotype*,#		
HLA-DR	↑ classical	=
CD86	↑ classical	=
CCR2	↑ classical, intermediate	=
CD11b	↑ non-classical	=
CD62L	=	↑ non-classical
CD51/CD61	↑ intermediate	↓ classical ↑ intermediate
RANK	↑ intermediate, non-classical	↑ intermediate
Serum RANKL/OPG		
sRANKL	↑	=
OPG	=	=
sRANKL/OPG	↑	=
Osteoclast differentiation and activity‡		
Osteoclast number	=	=
Number of nuclei/osteoclast	=	=
Pit number	=	=
Resorbed area	↑ d14, d21	=
Resorbed area/pit	↑ d21	=

* Assessed by flow cytometry; # Surface marker expression assessed by median fluorescence intensity; ‡ In vitro differentiation from circulating osteoclast precursors with macrophage-colony stimulating factor (M-CSF), receptor activator of nuclear factor-κB (RANK) ligand (RANKL), dexamethasone and transforming growth factor β (TGF-β) on bovine bone slices for 21 days. HLA - human leukocyte antigen; CD - cluster of differentiation; CCR - C-C chemokine receptor; OPG - osteoprotegerin; d - culture day.

We were particularly interested in the finding that the vitronectin receptor (CD51/CD61, $\alpha_v\beta_3$ integrin, essential for OC adhesion to bone matrix) expression was reduced in the classical subpopulation of AS patients, when compared to controls and in the non-classic subpopulation, when compared to RA, indicating that these cells might have a deficit in bone matrix attachment. There are only very few studies that analyze the phenotype of monocytes in AS patients. In one study, authors showed that CD11b was increased in patients with active AS when compared to AS patients with a BASDAI lower than 4; however no differences were found when compared to healthy donors [583]. Another study reported that monocyte derived dendritic cells from patients with active AS have lower HLA-DR expression when compared to controls, but this was not a direct assessment of monocyte phenotype [617].

Table 4 - Comparison between RA and AS patients of the monocytes subpopulation characteristics and osteoclast differentiation and activity

Parameter	RA	AS	p-value	corrected p-value†
Classic phenotype				
CCR2 MFI	139 [77-423]	76 [56-364]	0.001*	0.047*
HLA-DR MFI	282 [180-480]	226 [106-474]	0.035*	0.180
CD86 MFI	199 [124-478]	152 [99-288]	0.022*	0.043*
Intermediate phenotype				
CCR2 MFI	138 [85-335]	77 [8-542]	0.008*	0.444
CD86 MFI	366 [113-521]	254 [110-429]	0.024*	0.953
Non-classic phenotype				
CD86 MFI	336 [98-578]	219 [137-491]	0.049*	0.191
CD51/CD61 MFI	229 [118-757]	153 [14-430]	0.013*	0.035*
RANK MFI	183 [122-267]	143 [111-279]	0.024*	0.126
Osteoclast differentiation and activity				
pit number/mm^2 d14	65 [62-184]	27 [7-84]	0.023*	0.005*
resorbed area/pit d14 (μm^2)	5453 [756-5923]	443 [95-1812]	0.049*	0.020*
resorbed area/pit d21 (μm^2)	5471 [2058-18250]	1253 [156-4906]	0.027*	0.131
% resorbed area d14	35 [13-37]	1 [0-14]	0.004*	0.001*

Data is presented as median and range. AS - ankylosing spondylitis; CCR2 - C-C chemokine receptor type 2; CD - cluster of differentiation; d - culture day; HLA - human leukocyte antigen; MFI - median fluorescence intensity; RA - rheumatoid arthritis; RANKL - receptor activator of nuclear factor-κB. † - corrected for symptom duration, * - statistically significant.

Regarding circulating sRANKL and OPG levels we found that RA patients had increased levels of sRANKL and consequently increased sRANKL/OPG ratio when compared to the corresponding healthy donors, as previously described [618], but no differences were detected between AS patients and controls or between the two disease groups. Previous studies have shown discrepancies in sRANKL and OPG levels in AS patients [514, 619, 620]. Taylan *et al.* [514] included 55 AS Turkish patients treated only with NSAID and Klingberg *et al*. included 204 Swedish patients that could be treated with any therapy - 30% of patients were receiving DMARDs and 21% were receiving TNFi [620]. In these

studies authors found lower OPG, with normal sRANKL levels in AS patients, but they did not compare untreated patients with controls. However, in a study by Chen and colleagues in 55 AS Chinese patients the authors found that both sRANKL and OPG levels were higher in untreated patients [619]

OC precursor cells from RA patients formed the same number of OC as healthy donors but had higher activity, translated by an increased total resorbed area and higher resorbed area per lacunae. On the contrary, OC precursor cells from AS patients formed the same number of OC, with same activity as those from healthy donors. When we compared the two groups of patients, we observed that there was a transient increase in the number of OC and OC activity at culture day 14 that was still significant after we corrected for symptoms duration, confirming that cells from RA patients are more responsive to pro-osteoclastogenic stimuli. This observation is consistent with the study performed by Gengenbacher and colleagues that showed lower bone resorption in vitro in AS, when compared to RA patients [390]. To further understand the apparent non-responsiveness of AS circulating precursors we studied the expression of genes in the differentiated cells and found that on the first day, after attachment, without any stimuli, the expression of OC key genes as RANK, CSF1R (M-CSF receptor) and NFATc1 (an important osteoclast transcription factor) was lower than in healthy controls. This was also true on the last culture day, where we observed that both CSF1R and NFATc1 were still lower than in healthy donors. The same observation was also valid for the genes that code for ATPase, H^+ transporting, lysosomal V0 subunit D2 and cathepsin K, all relevant for OC resorption activity. These findings support our hypothesis that OC from AS patients have impairment in their activity when compared both to RA patients or healthy controls. This difference can be partially explained by an intrinsic inability to respond to osteoclastogenic stimuli and by downregulation of key OC differentiation and activity genes in AS patients.

Conclusions

The study of circulating OC precursors in RA and AS has showed that: 1) The cytokine pattern in early RA is promoting Th17 differentiation and osteoclastogenesis and it is not affected by either corticosteroids or MTX therapy. 2) In RA patients, MTX therapy is not efficient in decreasing OC formation and activity from circulating precursors. 3) TNFi decreases osteoclastogenesis and OC activity from circulating precursors from RA patients by downregulating key osteoclastogenic genes. 4) Monocytes from AS patients are not primed to respond to osteoclastogenic stimulus and have decreased OC specific gene expression despite high pro-inflammatory cytokine levels. 5) In AS patients, circulating OC precursors have decreased OC gene expression that is further inhibited by TNFi therapy; however, when stimulated to differentiate in OC, these precursors are very responsive to the stimuli suggesting an epigenetic mechanism that impairs osteoclastogenesis and is removed after TNFi therapy.

Although RA and AS are two chronic immune mediated diseases their effect on bone metabolism is different. The results of our evaluations of RA and AS patients indeed suggested that OC from AS patients have an impairment in activity despite the inflammatory activity of the disease. We propose that the differences we found between RA and AS OC circulating precursors are the result of a disease specific downregulation of key OC differentiation and activity genes in AS patients leading to the impairment of response to osteoclastogenic stimuli.

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Appendix

arthritis research&therapy

RESEARCH ARTICLE Open Access

Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis

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Abstract

Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by sustained synovitis. Recently, several studies have proposed neutrophils and Th17 cells as key players in the onset and perpetuation of this disease. The main goal of this work was to determine whether cytokines driving neutrophil and Th17 activation are dysregulated in very early rheumatoid arthritis patients with less than 6 weeks of disease duration and before treatment (VERA).

Methods: Cytokines related to neutrophil and Th17 activation were quantified in the serum of VERA and established RA patients and compared with other very early arthritis (VEA) and healthy controls. Synovial fluid (SF) from RA and osteoarthritis (OA) patients was also analyzed.

Results: VERA patients had increased serum levels of cytokines promoting Th17 polarization (IL-1β and IL-6), as well as IL-8 and Th17-derived cytokines (IL-17A and IL-22) known to induce neutrophil-mediated inflammation. In established RA this pattern is more evident within the SF. Early treatment with methotrexate or corticosteroids led to clinical improvement but without an impact on the cytokine pattern.

Conclusions: VERA patients already display increased levels of cytokines related with Th17 polarization and neutrophil recruitment and activation, a dysregulation also found in SF of established RA. 0 Thus, our data suggest that a cytokine-milieu favoring Th17 and neutrophil activity is an early event in RA pathogenesis.

Introduction

Rheumatoid arthritis (RA), the most common chronic autoimmune disease, affects approximately 1% of the population worldwide. This disease comprises a syndrome of pain, stiffness, and symmetrical synovitis which leads to joint destruction, functional disability, and substantial comorbidity due to the involvement of multiple organs and systems. The migration of leukocytes toward the synovium is crucial for the establishment of a chronic inflammatory process in RA [1-3]. This multi-regulated mechanism involves interactions with endothelial cells through cell adhesion molecules and complex cytokine and chemokine pathways.

Neutrophils specifically play an important role in the onset and perpetuation of RA, not only as interleukin (IL)-producing cells but also as cells responsible for the release of high amounts of reactive oxygen species and destructive enzymes, such as metalloproteases, contributing to joint erosions [4]. Neutrophils are among the first leukocytes to arrive at sites of inflammation. In fact, these cells are the most abundant in the synovial fluid (SF) of patients with active RA, and previous results from our group showed that the synovial tissue is heavily infiltrated by neutrophils in the first weeks of RA onset [5]. Interestingly, in animal models of arthritis, neutrophil depletion prevented joint inflammation if neutrophil-depleting antibodies were given before the

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induction of arthritis. Moreover, when the depleting antibody was given very early after the induction of arthritis, complete abrogation of the inflammatory symptoms was achieved [6].

T helper 17 (Th17) cells have also been proposed to have a relevant role in the early phase of RA through the production of IL-17 [7,8]. This cytokine promotes the recruitment and survival of neutrophils, induces the secretion of proinflammatory cytokines and the upregulation of RANKL (receptor activator of nuclear factor-kappa B ligand), and stimulates the activity of matrix metalloproteases, leading to cartilage catabolism and bone resorption [9,10]. The recruitment, activation, and effector function of Th17 cells and neutrophils are driven by a network of cytokines and chemokines secreted by multiple cellular sources. In established RA, it has been reported that IL-1β, IL-6, IL-8, IL-17, and tumor necrosis factor are elevated in the serum and this correlates with a higher disease activity [11-13]. Nevertheless, our knowledge of the influence of the cytokine network on RA onset remains limited. The characterization of the cytokine profile at this stage, where the transition from an acute to a chronic inflammatory phase occurs, may lead to the identification of early key players, with potential implications for early treatment strategies.

Thus, the main goal of our work was to determine whether cytokines driving neutrophil and Th17 cell activation and proinflammatory function were already present in very early RA (with less than 6 weeks of disease duration) and how this early cytokine environment differs from established RA. We also evaluated whether the introduction of low-dose corticosteroids and methotrexate (MTX) therapy had any influence on the cytokine profile observed at that early stage of the disease. We found that cytokines related to Th17 polarization and neutrophil recruitment and activation were elevated in early RA and that the conventional therapeutic options, though able to control clinical manifestations of the disease, were ineffective in reversing this underlying proinflammatory drive.

Materials and methods

Patients

Blood samples were obtained from 38 consecutive untreated polyarthritis patients with less than 6 weeks of disease duration. Some of these patients (19), after a minimum follow-up of 3 months, fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA [14]. These patients were classified as very early rheumatoid arthritis (VERA) patients, and further samples were collected 4 to 6 weeks after starting a low dose of oral corticosteroids (5 to 10 mg of prednisone) (time 1) and 4 months after reaching the minimum effective dose of MTX (time 2) (up to a maximum of 20 mg/week) that was required to reduce the disease activity score using 28 joint counts (DAS28) to less than 3.2 [15]. The remaining early arthritis patients (19), who did not develop RA, were classified as very early arthritis (VEA). Baseline blood samples from VERA and VEA patients were compared with 27 healthy donors used as controls. Additionally, 12 blood and 15 SF samples were obtained from patients with established RA. SF samples were also collected from 10 patients with osteoarthritis (OA) (Rheumatology Department, Hospital de Santa Maria, Lisbon, Portugal) (Table 1). Owing to the clinical characteristics of the VEA patients, SF in easily accessible joints was not available in VERA and VEA patients and thus SF was not analyzed in these groups of patients. The health assessment questionnaire (HAQ) [16] and DAS28 were applied to all patients. The study was approved by the local ethics committee, and all patients signed an informed consent form. Patient care was conducted in accordance with standard clinical practice, and the study was performed in accordance with the Declaration of Helsinki as amended in Edinburgh (2000).

Cytokine quantification

IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17A, IL-22, IL-23, and interferon-gamma levels were measured in the serum and SF by FlowCytomix assay kit (Bender MedSystems, Vienna, Austria) in accordance with the instructions of the manufacturer. Standard curves for each cytokine were generated by using reference cytokine concentrations supplied by the manufacturer. Samples were acquired with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Raw data of the flow cytometry bead assay were analyzed by FlowCytomix Pro 2.2 software (Bender MedSystems).

Measurement of autoantibodies

Rheumatoid factor (RF)-IgM was determined in all patients by means of an IMTEC Autoimmune Diagnostics ELISA [enzyme-linked immunosorbent assay] kit (Human GmbH, Wiesbaden, Germany) in accordance with instructions of the manufacturer, and samples were processed using a ChemWell 2910 automated analyzer (GMI, Ramsey, Minnesota, USA). Serum levels of anti-cyclic citrullinated peptide (anti-CCP) were measured by ELIA™ CCP test system (Phadia GmbH, Freiburg, Germany), and samples were analyzed with an ImmunoCAP 100 instrument (Phadia GmbH).

Statistical analysis

Statistical differences were determined with non-parametric Kruskal-Wallis, Mann-Whitney, and Wilcoxon signed-rank tests and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Correlation analysis was performed with the Spearman test.

Table 1 Clinical information about healthy controls and patients with VERA, VEA, RA, or OA

	Controls (*n* = 24)	VERA (*n* = 19)			VEA (*n* = 19)	RA (*n* = 12)	RA SF (*n* = 15)	OA SF (*n* = 10)
		Baseline	Time 1	Time 2				
Age in years, mean ± SD	40 ± 13		50 ± 17		40 ± 13	63 ± 10	57 ± 10	67 ± 13
Sex, female/male	17/7		16/3		15/4	11/1	11/4	5/5
DAS28, mean ± SD	NA	6.1 ± 1.8	4.1 ± 1.6[a]	3.1 ± 1.6[a]	4.5 ± 1.6[a]	5.2 ± 1.0	4.6 ± 1.4	NA
HAQ, mean ± SD	NA	1.4 ± 0.8	0.8 ± 0.7[a]	0.8 ± 0.7	0.8 ± 0.6[a]	1.5 ± 1.0	1.4 ± 0.8	NA
RF-positive, %	ND	42	ND	ND	0	67	ND	ND
Anti-CCP-positive, %	ND	32	ND	ND	0	45	ND	ND

[a]Disease activity score using 28 joint counts (DAS28) and health assessment questionnaire (HAQ) values were compared between very early rheumatoid arthritis (VERA) and very early arthritis (VEA) patients with reference to VERA baseline values. Differences were considered statistically significant for *P* values of less than 0.05. anti-CCP, anti-cyclic citrullinated peptide; IL, interleukin; MTX, methotrexate; NA, not applicable; ND, not determined; OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; SD, standard deviation; SF, synovial fluid.

Differences were considered statistically significant for *P* values of less than 0.05.

Results

Characterization of patients and disease evaluation

A total of 38 polyarthritis patients with less than 6 weeks of disease duration were evaluated. Nineteen patients fulfilled the 1987 ACR criteria for RA after a minimum follow-up of 3 months and were classified as VERA patients. The mean age of the VERA patients was 59 ± 17 years, 84% were female, 42% were RF-positive and 32% anti-CCP-positive, the initial DAS28 was 6.1 ± 1.8, and the initial HAQ was 1.4 ± 0.8. After treatment with low doses of prednisone and MTX, there was a significant reduction of both DAS28 and HAQ values (Table 1). The group of VEA patients included 19 patients, and 14 of them later had one of the following diagnoses: spondylarthritis (5 cases), systemic lupus erythematosus (4 cases), crystal induced arthritis (2 cases), Sjögren syndrome (1 case), paraneoplastic polyarthritis related to multiple myeloma (1 case), and arthritis associated with HIV infection (1 case). Five patients entered spontaneously into remission before 3 months of follow-up, remaining without a specific diagnosis and were thus classified as presenting a self-limited form of polyarthritis. The mean age of the VEA patients was 40 ± 13 years, 79% were female, all patients were RF-negative and anti-CCP-negative, the initial DAS28 was 4.5 ± 1.6, and the initial HAQ was 0.8 ± 0.6. Both DAS28 and HAQ values were significantly lower than those of VERA patients at baseline (Table 1). These early polyarthritis patients represent a subset of a larger cohort previously described by our group [17].

Furthermore, blood samples were collected from 12 patients with established RA; mean age was 60 ± 10 years, 92% were female, and 67% were RF-positive and 45% anti-CCP-positive (Table 1). Additionally, SF samples were collected from 12 patients with established RA; mean age was 57 ± 10 years, and 73% were female (Table 1). The established RA group of patients had a DAS28 and a HAQ mean scores similar to VERA baseline values.

IL-8 is increased in VERA patients and locally in the joints of patients with established RA

Given the proposed role of neutrophils in the pathogenesis of RA [18,19], we quantified the major neutrophil chemoattractant, IL-8, in the serum of VERA patients. At baseline, VERA patients had significantly higher levels of IL-8 when compared with both VEA and healthy controls (Figure 1a). After 2 to 4 weeks of low-dose corticosteroids and after 4 months of MTX therapy, there were no significant changes in the levels of circulating IL-8 (data not shown). Interestingly, VERA patients also had significantly higher circulating levels of IL-8 in comparison with serum from established RA (Figure 1a). Neutrophils accumulate locally in the joints of patients with RA [20]. Thus, we quantified the concentration of IL-8 in the SF of patients with RA and compared the concentration with that of SF from patients with OA. We found significantly higher levels of IL-8 in the SF of patients with RA in comparison with OA SF (Figure 1b).

IL-17 levels are dysregulated in both VERA patients and patients with established RA

Previous studies from our group showed that there is a delay in the apoptosis of circulating neutrophils in VERA patients [21]. Therefore, we analyzed IL-17A levels in these patients since it has already been described that this cytokine is important for the survival of neutrophils [22]. Moreover, IL-17A is a signature cytokine of Th17 cells, a subset proposed to have a key role in RA pathogenesis [9,23]. We found that VERA patients had significantly higher levels of IL-17A when compared with healthy controls, but not with VEA patients (Figure 2a). Furthermore, in our previous work, we found no difference in the frequency and absolute

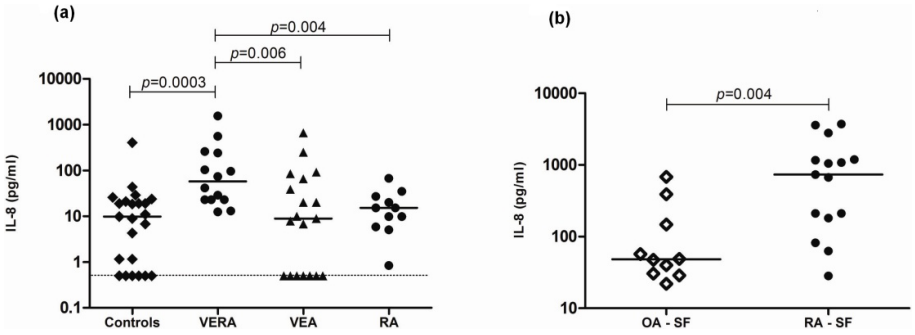

Figure 1 Interleukin-8 (IL-8) is increased in the serum of very early rheumatoid arthritis (VERA) patients and in synovial fluid (SF) of established rheumatoid arthritis (RA). **(a)** The serum concentration of IL-8 was measured in VERA and very early arthritis (VEA) patients as well as healthy controls and patients with established RA. The serum concentration of IL-8 was increased in VERA patients compared with any other group. Dotted line represents the limit of detection for the assay. **(b)** The concentration of IL-8 was measured in the SF collected from patients with established RA and from a control group with osteoarthritis (OA). We found a significant increase of IL-8 in RA-SF. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

numbers of $CD4^+$ and $CD8^+$ T-cell subpopulations in the peripheral blood of these patients when analyzed by flow cytometry [17].

Regarding the effects of early therapy, we found that neither corticosteroids nor MTX affected the level of IL-17A (data not shown). Moreover, IL-17A was significantly increased locally within the joints of patients with established RA in comparison with control SF from patients with OA (Figure 2b).

RA has a Th17-cytokine pattern since the very first weeks of onset

Having found that IL-17A was elevated in VERA patients, we decided to quantify a panel of cytokines known to be associated with Th17 polarization. At baseline, VERA patients had significantly higher levels of IL-1β and IL-22 in comparison with both VEA and healthy controls. In addition, we found that VERA patients have significantly higher IL-6 levels than healthy controls (Figure 3). Furthermore, the significantly higher circulating levels of IL-6 and IL-22 were maintained in established RA (Figure 3).

Locally, within the joints of patients with RA, the SF displayed elevated levels of IL-1β and IL-6 in comparison with OA SF (Figure 4 and Table 2). Moreover, no significant differences could be observed for IL-23 in circulation or locally in the joints (data not shown). We have also studied cytokines associated with the function of Th2 (IL-4 and IL-10) and Th1 (IL-2, IL-12 (p70), and INFγ) cells. However, no statistically significant differences could be observed for any of these cytokines (data not shown).

Discussion

Several studies have previously demonstrated that neutrophils play an important role in the onset of RA [21].

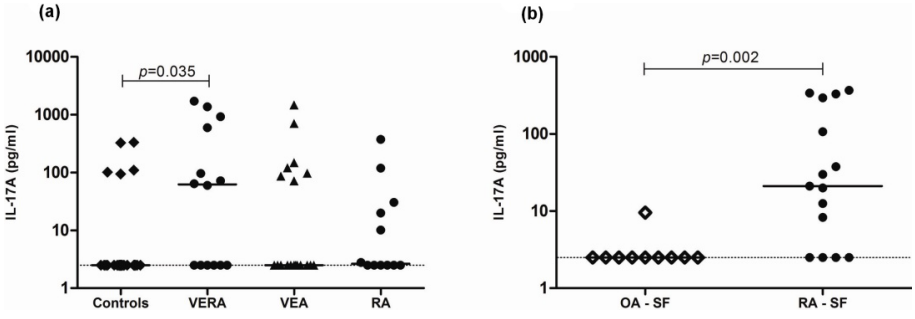

Figure 2 Very early rheumatoid arthritis (VERA) patients and synovial fluid (SF) of established rheumatoid arthritis (RA) display increased levels of interleukin-17A (IL-17A). **(a)** The serum concentration of IL-17A was measured in VERA and very early arthritis (VEA) patients as well as healthy controls and patients with established RA. The serum concentration of IL-17A was increased in VERA patients compared with healthy controls. **(b)** The concentration of IL-17A was measured in the SF collected from patients with established RA and from a control group with osteoarthritis (OA). In the SF of patients with RA, we observed a significant increase of IL-17A. Dotted lines represent the limit of detection for the assay. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

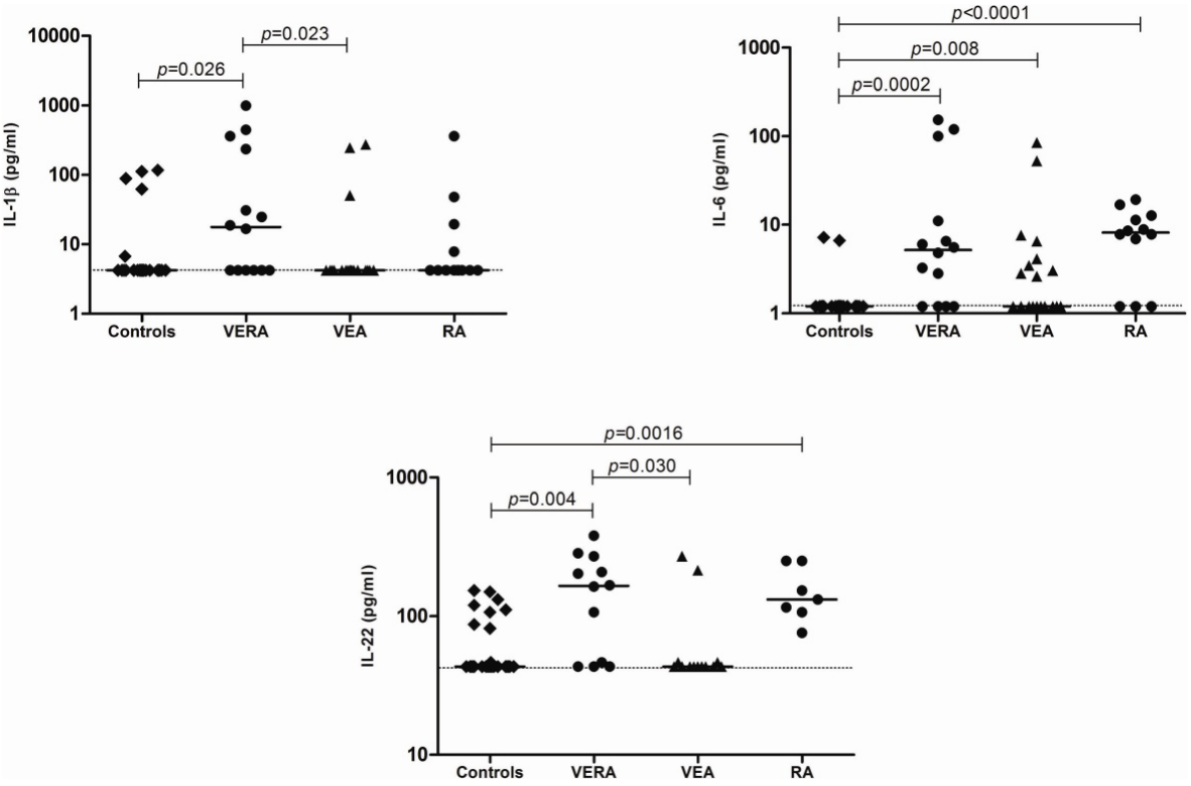

Figure 3 Cytokines related to T helper 17 (Th17) polarization are increased in the serum of very early rheumatoid arthritis (VERA) patients and synovial fluid of established rheumatoid arthritis (RA). The serum concentrations of interleukin (IL)-1β, IL-6, and IL-22 were measured in VERA and very early arthritis (VEA) patients as well as healthy controls and patients with established RA. All three cytokines were increased in VERA patients compared with healthy controls. IL-6 was equally elevated in all groups of patients with an inflammatory disease, whereas the other two cytokines were increased only in VERA (IL-1β) or in VERA and RA patients (IL-22). Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

This hypothesis is supported by data from animal models [24]. In fact, neutrophils are the most abundant leukocytes in the SF of patients with active RA, and in early RA, these cells show significantly lower levels of apoptosis when compared with patients with other persistent forms of arthritis or with arthritis that has a self-limited disease course [25]. Additionally, previous results from our group demonstrated that there is a delay in the apoptosis of circulating neutrophils in VERA patients [21] and that these cells heavily infiltrate the synovial tissue during RA onset [5].

In the present study, we demonstrate that a neutrophil- and Th17-driving cytokine pattern is present in untreated VERA patients with less than 6 weeks of

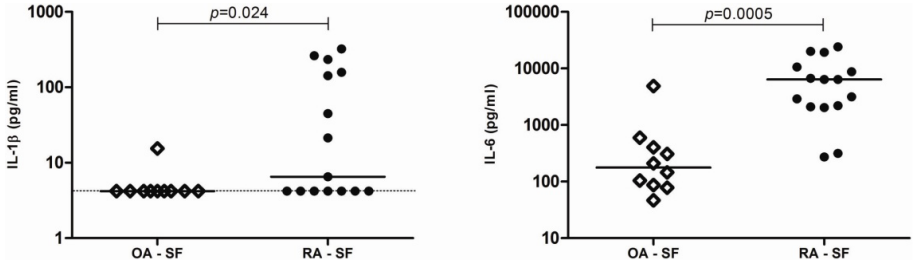

Figure 4 Cytokines related to T helper 17 (Th17) polarization are increased in the synovial fluid (SF) of established rheumatoid arthritis (RA). The concentrations of interleukin (IL)-1β and IL-6 were markedly increased in the SF collected from patients with established RA when compared with osteoarthritis (OA). Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

Table 2 Cytokine levels in healthy controls and patients with VERA, VEA, established RA, or OA

Cytokine, pg/mL	Controls	VERA	VEA	RA	RA SF	OA SF
IL-1β	4.2 (4.2-116.8)	17.7 (4.2-99.7)	4.2 (4.2-272.7)	4.2 (4.2-360.3)	6.5 (4.2-322.1)	4.2 (4.2-15.5)
IL-6	1.2 (1.2-7.2)	5.2 (1.2-153.2)	1.2 (1.2-84.7)	8.2 (1.2-19.7)	6361.0 (272.7-24,135.0)	177.6 (46.6-4,881.0)
IL-8	9.9 (0.5-407.7)	57.6 (12.5-1,546.0)	8.9 (0.5-665.2)	15.2 (0.8-67.5)	735.7 (28.3-3,717.0)	48.2 (22.0-680.0)
IL-17A	2.5 (2.5-333.7)	62.0 (2.5-1,714.0)	2.5 (2.5-1,477.0)	2.6 (2.5-375.6)	21.1 (2.5-369.1)	2.5 (2.5-9.5)
IL-22	43.3 (43.3-153.4)	165.3 (43.3-380.6)	43.3 (43.3-270.5)	131.7 (75.8-250.7)	153.4 (75.8-336.0)	151.7 (92.4-235.3)

Values are presented as median (range). IL, interleukin; OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial fluid; VEA, very early arthritis; VERA, very early rheumatoid arthritis.

disease duration. We consider this observation of interest because the knowledge concerning the immune mechanisms associated with the onset of RA is still elusive. In fact, the majority of early RA studies include patients with 3 to 12 months of disease duration or even more. In accordance with an early participation of neutrophils in RA, our results revealed that VERA patients have increased levels of IL-8 when compared with both VEA and healthy controls, and this could explain the preactivated state of circulating neutrophils [18] and their recruitment toward the SF from the very first weeks of RA onset.

In addition, Th17 cells are known to be important for the promotion of neutrophil-mediated inflammation by producing IL-17A, a cytokine known to indirectly activate neutrophil chemotaxis and extend their survival [10,22]. We found a high serum concentration of IL-17A in VERA patients as well as locally within the joints of patients with established RA. This might indicate that an activation of Th17 cells from a very early phase of the disease can promote neutrophil participation in RA pathogenesis [22]. However, we found no evidence for changes in the frequency of T-cell subsets in the peripheral blood of VERA patients [17]. This observation is not unexpected; the relatively small representation of antigen-specific T cells in the circulating pool are the activated T cells that drive the pathology more likely found within the tissues [26]. In a study performed by Kokkonen and colleagues [27], the levels of several cytokines and chemokines were analyzed in blood samples from a group of individuals 3.3 years before RA onset ('pre-patients') and compared with healthy donors and RA patients with 7.7 ± 3.6 months of disease duration. An interesting finding was that IL-17 was present at its highest concentration in pre-patients and the level of this cytokine was lower in patients with RA. This is in accordance with our own results; we observed an increased level of IL-17 in RA patients with less than 6 weeks of disease duration, whereas in patients with established RA, the levels were not significantly different from those of healthy controls. Remarkably, the IL-17 median concentration observed in our established RA cohort (2.6 pg/mL) was even lower than that of RA patients from the work of Kokkonen and colleagues [27] (6.0 pg/mL). Thus, this observation reinforces the role of IL-17 in the initial phase of RA, and as the pathogenesis progresses to a chronic stage, other factors are subsequently brought into action in the peripheral blood. Unlike Kokkonen and colleagues, we have not detected differences in Th1- and Th2-related cytokines between both VERA and patients with established RA in comparison with controls. These discrepancies might be related to the different methodologies used.

Additionally, the elevated levels of IL-1β observed in VERA patients can stimulate endothelial cells, T and B cells, and fibroblasts in the joints to produce IL-6 and IL-8. But importantly, IL-1β and IL-6, both found to be increased in VERA patients, are known to promote the differentiation of Th17 cells, which in turn secrete IL-17A and IL-22 [28,29], two cytokines that were elevated in VERA patients and have an essential function in the pathogenesis of autoimmune diseases [29].

Currently, the treatment of choice for RA at the time of presentation is MTX. Interestingly, in spite of clinical improvement (DAS28 reduced from 6.1 ± 1.8 to 3.1 ± 1.6), neither therapy with low-dose corticosteroids nor combined therapy with low-dose corticosteroids and MTX corrected the dysregulated cytokine pattern observed in VERA patients. In fact, low-dose corticosteroids and MTX have unclear effects on the RA cytokine network. For instance, corticosteroids fail to reduce serum levels of IL-1β and IL-8 [30] and MTX does not alter serum IL-1β concentration when compared with pre-treatment levels [31,32]. Our results suggest that the conditions contributing to Th17 cells and neutrophil-mediated inflammation, thus driving early pathogenesis, are not modified with early treatment with low-dose corticosteroids and MTX.

The elevated IL-1β, IL-6, IL-8, and IL-17A levels observed in the SF of patients with RA confirm a local

role for these cytokines in the maintenance of synovitis. Moreover, IL-6 can support a continuous recruitment of autoreactive B cells toward the synovium [33,34], contributing to an exacerbation of the inflammatory process because of the production of autoantibodies and immune complexes.

Conclusions

Taken together, our data reinforce the potential relevance of therapies targeting IL-1β [35,36] and IL-6 [37,38] in early RA. In addition, the data establish IL-8 and IL-17A as other potential therapeutic targets at an early stage of the disease. Finally, we found that MTX and corticosteroids, though effective in reducing disease activity in VERA patients, do not appear to correct underlying cytokine dysregulation driving the Th17/neutrophil-mediated inflammation.

Abbreviations

ACR: American College of Rheumatology; anti-CCP: anti-cyclic citrullinated peptide; DAS28: disease activity score using 28 joint counts; HAQ: health assessment questionnaire; IL: interleukin; MTX: methotrexate; OA: osteoarthritis; RA: rheumatoid arthritis; RF: rheumatoid factor; SF: synovial fluid; Th17: T helper 17; VEA: very early arthritis; VERA: very early rheumatoid arthritis.

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Authors' contributions

RC and RAM equally performed all of the laboratorial work, data collection, and statistical analysis and wrote the paper. IP contributed to some of the laboratory experiments. HC, ES, AFM, AMR, and JP-P were responsible for the selection, follow-up, and medical care of patients enrolled in this study and helped review the paper. MVQ participated as the head of the Rheumatology Department of Hospital de Santa Maria, which approved the study and patients' management. HSR and MMS-C made a substantial intellectual contribution to the present work and revised it critically. LG and JEF, as senior authors, conceived of the study, participated in its design and coordination, and contributed important intellectual input to the draft of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Ankylosing Spondylitis

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Abstract

Introduction

Ankylosing Spondylitis (AS) is characterized by excessive local bone formation and concomitant systemic bone loss. Tumor necrosis factor (TNF) plays a central role in the inflammation of axial skeleton and enthesis of AS patients. Despite reduction of inflammation and systemic bone loss, AS patients treated with TNF inhibitors (TNFi) have ongoing local bone formation. The aim of this study was to assess the effect of TNFi in the differentiation and activity of osteoclasts (OC) in AS patients.

Methods

13 AS patients treated with TNFi were analyzed at baseline and after a minimum follow-up period of 6 months. 25 healthy donors were recruited as controls. Blood samples were collected to assess receptor activator of nuclear factor kappa-B ligand (RANKL) surface expression on circulating leukocytes and frequency and phenotype of monocyte subpopulations. Quantification of serum levels of bone turnover markers and cytokines, *in vitro* OC differentiation assay and qRT-PCR for OC specific genes were performed.

Results

$RANKL^+$ circulating lymphocytes (B and T cells) and IL-17A, IL-23 and TGF-β levels were decreased after TNFi treatment. We found no differences in the frequency of the different monocyte subpopulations, however, we found decreased expression of CCR2 and increased expression of CD62L after TNFi treatment. OC number was reduced in patients at baseline when compared to controls. OC specific gene expression was reduced in circulating OC precursors after TNFi treatment. However, when cultured in OC differentiating

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conditions, OC precursors from AS TNFi-treated patients showed increased activity as compared to baseline.

Conclusion

In AS patients, TNFi treatment reduces systemic pro osteoclastogenic stimuli. However, OC precursors from AS patients exposed to TNFi therapy have increased *in vitro* activity in response to osteoclastogenic stimuli.

Introduction

Ankylosing spondylitis (AS) is a systemic, chronic, immune-mediated inflammatory disease that affects the musculoskeletal system. The axial skeleton and enthesis are predominantly involved in this disease and tumor necrosis factor (TNF) seems to play a central role [1]. AS is characterized by local excessive bone formation, but it is also associated with systemic bone loss, which is a common complication even in the early stages of the disease [2].

The immune and skeletal systems have several common regulatory factors and immune system cells have a profound influence on bone metabolism, particularly in chronic inflammatory diseases. Receptor activator of nuclear factor κB ligand (RANKL) is present on osteoblasts surface, but is also expressed by activated immune cells, both in its membrane form and as a soluble molecule [3]. Cytokines such as TNF, interleukin (IL)-1β, IL-6 and IL-17 are secreted by activated immune cells and act synergistically with the RANK-RANKL system [4,5], further enhancing osteoclast (OC) differentiation from its circulatory precursors (monocytes) and contributing to bone resorption [1,3]. Monocytes are phenotypically and functionally heterogeneous and have a critical regulatory role in inflammation and innate immune responses [6]. Three sub-populations of monocytes have been described in humans, based on their expression of CD14 and CD16 surface markers. The classical subset, $CD14^{bright}CD16^{-}$ accounts for 85% of monocytes, includes phagocytic cells and OC precursors; the non-classical subset $CD14^{dim}CD16^{+}$ accounts for 10% of monocytes and is involved in cytokine production and T-cell activation. The intermediate, the most recently described subset, accounts for only 5% of monocytes and is $CD14^{bright}CD16^{+}$. This latter subset is considered to be the antigen presenting subset and is responsible for reactive oxygen species production [6]. Monocytes are key players in immune-mediated inflammatory diseases and their excessive and sustained activity is a hallmark of AS [7].

Serum levels of TNF, IL-6 and IL-17 are increased in AS patients, which may contribute to the well documented secondary osteoporosis that occur in these patients [1,8]. TNFi are very effective in the mitigation of inflammation in AS patients and induce a reduction in CTX-I levels, which may reflect a decrease in OC activity [8]. The aim of this study was to assess the effect of TNFi in the differentiation and activity of OC precursors in AS patients.

Patients and Methods

Patients

The local ethics committee (Hospital de Santa Maria) approved this study and all participants signed an informed consent. Patients were managed in accordance with the standard practice and the study was conducted in accordance with the Declaration of Helsinki as amended in Brazil (2013). Patients with AS fulfilling the New York modified criteria 1984 [9] were

recruited from the Rheumatology and Bone Metabolic Disease Department, Lisbon Academic Medical Centre, Portugal. All patients were included before starting the first TNFi and were followed-up during a minimum period of 6 months after initiating therapy. Other inclusion criteria at baseline were: active disease, defined as AS disease activity score (ASDAS-ESR)>1.3 [10] and documented axial involvement by X-ray or magnetic resonance imaging (MRI). Patients previously exposed to biological TNFi were excluded. Information regarding patients' demographics, duration of symptoms, peripheral involvement, syndesmophyte formation, HLA-B27 positivity, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) was collected. ASDAS was evaluated, as well as the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI [11]) and the Bath Ankylosing Spondylitis Functional Index (BASFI [12]). Heparinized blood and serum were collected from each participant at the starting date of TNFi and another collection was made after a minimum period of 6 months of follow-up. Blood was used for flow cytometry and peripheral blood mononuclear cell (PBMC) isolation. Donors matched for age and gender were used as controls. Samples were stored and managed by the Biobanco-IMM, Lisbon Academic Medical Center, Lisbon, Portugal. The local ethics committee approved this study and all participants signed an informed consent.

Flow cytometry

Identification of B and T cells and granulocytes in peripheral blood and immunophenotyping of monocytes in the PBMC samples were performed using matched combinations of anti-human murine mAbs. For peripheral blood staining anti-CD19 PerCP-Cy5.5 (eBioscience), anti-CD3 PerCP (BD Biosciences), anti-CD66b FITC (Immunotools) and anti-RANKL PE (Santa Cruz Biotechnology) were used. Monocyte subpopulations were identified with anti-CD14 FITC (BD Biosciences) or PerCPCy5.5 (Immunotools) and anti-CD16 APC (Immunotools) and stained with combinations of anti-CD11b PE-Cy7, CD105 PE, CD62L PE-Cy7, CD51/CD61 FITC (eBioscience), CCR2 PE (R&D Systems), HLA-DR PerCP (BD Biosciences) and RANK PE (Santa Cruz Biotechnology). Cell death was assessed by staining with Annexin V Apoptosis Detection Kit APC (eBioscience). Acquisition was performed using a FACSCalibur (BD Biosciences).

Heparinized whole blood was used for staining. Erythrocytes were lysed with red blood cell lysis buffer and cells were incubated with IgG block solution 300ng/mL (ChromPure Mouse IgG whole molecule, Jackson ImmunoResearch Laboratories) before staining. Absolute cell counts were calculated from differential leukocyte count determined for all participants. PBMCs were isolated by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich). Monocyte subpopulations were identified as described before based on their CD14 and CD16 surface expression [6]. Data was analyzed using FlowJo software (TreeStar, Stanford University).

Cytokine detection in the serum

IL-1β, IL-6, IL-12(p70), IL-17A, IL-23, monocyte chemotactic protein-1 (MCP-1), transforming growth factor-beta (TGF-β) and TNF levels were measured in the serum by FlowCytomix custom assay kits (Bender MedSystems) according to the manufacturer instructions. Samples were acquired with a FACS Calibur flow cytometer (BD Biosciences). Raw data of the flow cytometry bead assay were analyzed by FlowCytomix Pro 3.0 software (Bender MedSystems). Carboxy-terminal type I collagen crosslinks (CTX-I), human type I procollagen amino-terminal-propeptide (P1NP, Sunred Biological technology), osteoprotegerin (OPG), sclerostin (SOST), dickkopf-related protein (DKK)-1 and soluble RANKL (ampli-sRANKL, Biomedica

Grouppe) were quantified by enzyme-linked immunosorbent assay (ELISA) in serum samples according to the manufacturer's instructions.

PBMC isolation and cell culture

PBMCs were isolated by density gradient centrifugation and plated in 96-well culture plates at a density of $7.0x10^5$ cells/well and in 24-well culture plates at a density of $1.5x10^6$ cells/well in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5000 U Penicillin/Streptomicin (Invitrogen), 2 mM L-Glutamine (Invitrogen) and 10% Fetal Bovine Serum (FBS; Invitrogen) and incubated in a humidified atmosphere at 37°C, 5% CO_2. PBMCs were left overnight for OC precursors (OCPs) to adhere on bone slices. On the following day (day 1 of culture) medium was changed to DMEM supplemented with M-CSF 25 ng/mL (Peprotech) and three days later, medium was again changed to DMEM supplemented with M-CSF (25 ng/mL), sRANKL (50 ng/mL; Peprotech), dexamethasone (10 nM; Sigma Aldrich) and TGF-β (2.5 ng/mL; R&D Systems) in order to differentiate the osteoclast precursors into mature osteoclasts. The culture medium was then changed twice a week. Adherent cells at day 1 and cells cultured on bone slices for 7, 14 and 21 days [13] were used for functional assays and gene expression.

Functional assays

Tartrate-resistant acid phosphatase (TRAP) staining of OCs was performed at days 7, 14 and 21 of culture using the Acid Phosphate Leukocyte Kit (TRAP, Sigma-Aldrich) according to the manufacturer's instructions. OCs were counted as TRAP positive cells containing three or more nuclei [14,15]. For measurement of resorbed area in the resorption assay at days 7, 14 and 21 of culture, cells were removed from the bone slices using sodium hypochlorite and stained with 0.1% toluidine blue [16]. Bone slices from both TRAP staining and resorption functional assays were photographed in an area of 1.25 mm^2 with a brightfield microscope (Leica DM2500, Leica) under a 10x objective. The number of TRAP stained OCs was counted for each time-point per condition and the resorption pits were traced using ImageJ software (NIH, Bethesda, MD). The resorbed area was calculated and expressed in % of total area.

Gene expression

RNA was extracted from cells cultured over the bone slices at days 1, 7, 14 and 21 of culture using NZYol (NZYTech) according to the manufacturer's instructions. Following RNA extraction, total RNA concentration and purity was quantified using Nanodrop 1000 (Thermo Scientific). Complementary (c)DNA was synthesized at a concentration of 0.6 ng/μL using the DyNAmo™ cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Genes that encode for osteoclast proteins such as CSF1R, RANK, NFATc1, ATP6V0D2 and CTSK were studied (see Table 1 for primers) by real-time quantitative PCR (RT-qPCR). Ribossomal RNA 18s was chosen as the housekeeping gene. Primers were designed using the primer-BLAST [17] software and qPCR was performed using the DyNAmo™ Flash SYBR Green qPCR Kit (Thermo Scientific). The efficiency of qPCR was analysed using the standard curve method [18] as described previously [19]. The values obtained were normalized with the housekeeping gene 18s rRNA.

Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (IBM). Categorical variables were expressed as frequencies and comparisons were tested using chi-square test. Continuous

Table 1. Primers used for osteoclast gene expression.

Gene	Primer sequence	Transcript size
CFSR1	Fw 5'—GAACATCCACCTCGAGAAGAAA—3'	88bp
	Rev 5'—GACAGGCCTCATCTCCACAT—3'	
RANK	Fw 5'—GAACATCATGGGACAGAGAAATC—3'	89bp
	Rev 5'—GGCAAGTAAACATGGGGTTC—3'	
NFATc1	Fw 5'—GCAAGCCGAATTCTCTGGTG—3'	144bp
	Rev 5'—TACCGTTGGCGGGAAGGTAG—3'	
ATP6V2D0	Fw 5'—CATTCTTGAGTTTGAGGCCG—3'	186bp
	Rev 5'—CCGTAATGATCCGCTACGTT—3'	
CTSK	Fw 5'—GCCAGACAACAGATTTCCATC—3'	75bp
	Rev 5'—CAGAGCAAAGCTCACCACAG—3'	
18s rRNA	Fw 5'—GGAGTATGGTTGCAAAGCTGA—3'	129bp
	Rev 5'—ATCTGTCAATCCTGTCCGTGT—3'	

Annealing temperature for all primers was 60°C

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variables were expressed by median and interquartile range. Baseline and post-treatment (follow-up) values within each sample were compared using Wilcoxon's matched-pairs signed-rank test. To compare AS patients with healthy age and sex-matched donors Mann-Whitney test was used. Correlation analysis was performed using Spearman's correlation coefficients. Values were corrected for multiple comparisons and p-values lower than 0.05 were considered significant.

Results

Patient background

Thirty-eight subjects were recruited, including 13 AS patients, evaluated before and after TNFi therapy, and 25 age and gender matched healthy donors. Despite having an initial cohort of 25 patients 3 were lost for follow-up and 9 switched biological therapy at 3 months follow-up. Patients were treated with one of the four TNFi currently used in clinical practice: Adalimumab (n = 1, 8%), Golimumab (n = 5, 38.5%), Infliximab (n = 2, 15%) or Etanercept (n = 5, 38.5%). Treatment duration ranged from a minimum of 6 up to 12 months. The clinical and demographic characteristics of the patients both at baseline and follow-up and healthy donors are described in Table 2.

TNFi treatment in AS patients decreases the number of RANKL$^+$ T and B cells in circulation

RANKL surface staining was performed in whole blood leukocytes (neutrophils—CD66b$^+$; T cells CD3$^+$; B cells CD19$^+$; Fig 1A). No difference was found in the total number of circulating neutrophils, T or B cells before or after therapy or when compared to healthy donors (data not shown). However, CD66b$^+$RANKL$^+$ cells were higher in patients than in healthy donors, both at baseline and follow-up. After TNFi therapy patients had lower number of CD3$^+$RANKL$^+$ cells in circulation when compared to healthy donors, both in percentage and absolute number ($p = 0.0271$ and $p = 0.0244$, respectively; Fig 1A). Furthermore CD19$^+$RANKL$^+$ cell frequency and absolute number was decreased in patients after TNFi treatment (percentage value significantly different, $p = 0.0122$; Fig 1A).

Table 2. Summary of the patients and healthy controls' characteristics.

	AS patients		Healthy	p-value
	Baseline	Follow-up		
Sample size	13		25	
Age (years)	37 [33–43]		39 [36–49]	0.8028
Females %	38%		48%	0.7342
Symptoms duration (years)	10 [7–21]		NA	
HLA-B27 (% positive)	54%		NA	
Presence of syndesmophytes (%)	40%		NA	
Peripheral involvement (%)	46%		NA	
Treatment with NSAIDs (%)	77%		NA	
NSAIDs duration (months)	24 [8–42]		NA	
Treatment with DMARDs (%)	46%		NA	
DMARDs duration (months)	24 [7–47]		NA	
Treatment with corticosteroids (%)	15%		NA	
ESR (mm/h)	30 [14–54]	7 [4–17]	NA	0.0010*
CRP (mg/dl)	1.4 [0.1–3.0]	0.1 [0.0–0.6]	NA	0.0034*
ASDAS	3.8 [2.2–4.3]	1.7 [1.4–1.9]	NA	0.0001*
BASDAI	4.7 [3.9–7.5]	2.5 [1.5–4.1]	NA	0.0007*
BASFI	6.2 [5.1–7.4]	3.9 [1.2–5.4]	NA	0.0032*
TNFi duration (months)	NA	12 [6–12]	NA	

Data is represented as median [Interquartile range] unless stated otherwise; DMARDs include methotrexate, hydroxychloroquine and sulfasalazine; AS—ankylosing spondylitis; HLA—human leukocyte antigen; NA—not applicable; NSAIDs—non-steroidal anti-inflammatory drugs; DMARDs—disease-modifying antirheumatic drugs; ESR—erythrocyte sedimentation rate; CRP—C-reactive protein; ASDAS—ankylosing spondylitis disease activity score; BASDAI—Bath ankylosing spondylitis disease activity index; BASFI—Bath ankylosing spondylitis functional index; TNFi—tumor necrosis factor inhibitors.
* p-value<0.05.

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Regarding monocyte subpopulations (classical $CD14^{bright}CD16^{-}$, intermediate $CD14^{bright}CD16^{+}$ and non-classical $CD14^{dim}CD16^{+}$) no differences in frequency or cell death among the three subpopulations and between groups were found. CD62L, a cell adhesion molecule also known as L-selectin, was increased in the circulating classical subpopulation of patients, both at baseline and follow-up, when compared to healthy donors ($p = 0.0436$; $p = 0.0158$; Fig 1B). Moreover, comparing with healthy donors, CD62L expression was higher in patients after 6 months of TNFi therapy both in the non-classical subpopulation ($p = 0.0398$; Fig 1B) and in the intermediate ($p = 0.001$; Fig 1B) subpopulations. Conversely, CCR2 expression was lower in the intermediate subpopulation in patients after TNFi when compared to healthy donors ($p = 0.0307$; Fig 1B). No differences were identified in any of the other studied surface markers.

IL-17A, IL-23 and TGF- β circulating levels are reduced in AS patients after TNFi treatment

DKK-1, IL-1β, IL-6, IL-17A, IL-12p70, IL-23, TNF, MCP-1 and TGF-β levels were significantly higher in patients at baseline when compared to healthy donors (Fig 2). After correcting for multiple comparisons only IL-1β, IL-23, MCP-1 and TNF remained significantly higher in patients at baseline when compared to healthy donors.

Circulating levels of IL-17A, IL-23 and TGF-β were decreased after TNFi treatment when compared to baseline ($p = 0.0195$, $p = 0.0098$ and $p = 0.0115$, respectively; Fig 2). CTX-I levels

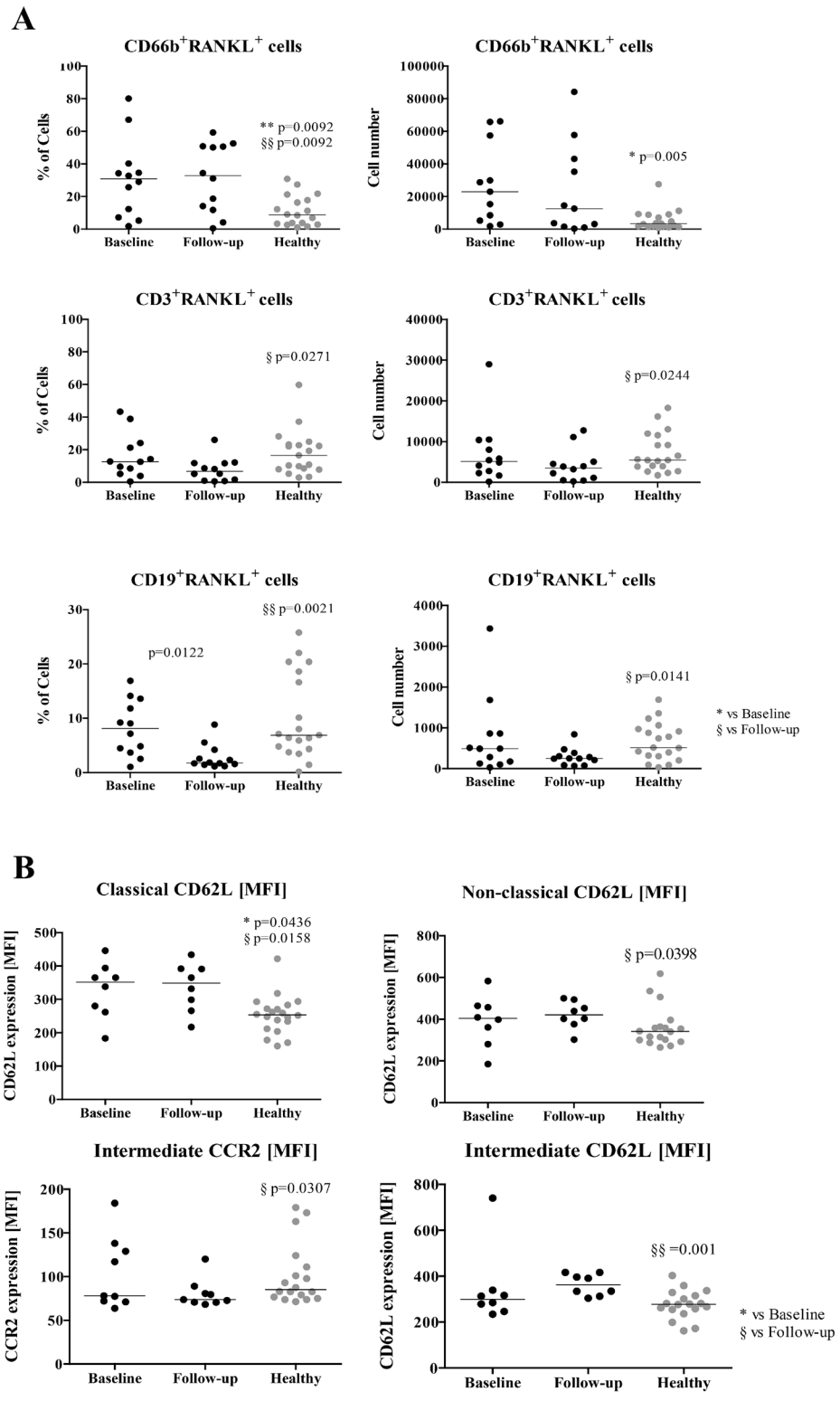

Fig 1. RANKL surface expression in leukocytes and monocyte phenotype. A) RANKL$^+$ frequency and absolute number in circulating leukocytes. CD66b$^+$RANKL$^+$ cells are increased in the circulation of patients at baseline and at follow-up when compared to healthy donors (frequency p = 0.0092; absolute number p = 0.005). At follow-up, CD3$^+$RANKL$^+$ are decreased in circulation when compared to healthy donors (frequency p = 0.0271; absolute number p = 0.0244). CD19$^+$RANKL$^+$ frequency is decreased after treatment

when compared to patients at baseline (p = 0.0122) and with healthy donors (p = 0.0021). This difference is also observed at the absolute number level when compared to healthy donors (p = 0.0141). $RANKL^+$ cells were analysed by flow cytometry and gated inside each subpopulation. B) Phenotype of circulating monocyte subpopulations—Classical $CD14^{bright}CD16^-$, intermediate $CD14^{bright}CD16^+$, non-classical $CD14^{dim}CD16^+$. CD62L is increased in the circulating classical subpopulation of patients at baseline (p = 0.0436) and at 6 months follow-up (p = 0.0158) when compared to healthy donors. CD62L expression is increased in patients after 6 months follow-up both in the non-classical subpopulation (compared to healthy p = 0.0398) and in the intermediate subpopulation (compared to healthy p = 0.001). CCR2 expression is reduced in the intermediate subpopulation of patients at follow-up when compared to healthy donors (p = 0.0307). Each dot represents a sample. Line represents median. * vs Baseline, § vs Follow-up. * and § p<0.05, ** and §§ p<0.01. MFI—Median fluorescence intensity.

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were lower in patients at 6 months of follow-up when compared to healthy donors (p = 0.0268; Fig 2). After correcting for multiple comparisons, none of the markers were significantly decreased after treatment.

Osteoclast differentiation from circulating precursors in AS patients is lower than in healthy controls and osteoclast-mediated bone resorption is increased after TNFi treatment

Under stimulation, reproducing local bone inflammatory microenvironment, AS patients, prior to TNFi, had less OC differentiation at culture day 21 than healthy donors (p = 0.0038; Fig 3A). However, the number of OC increased throughout the culture period in all the studied groups. No differences were found in the number of nuclei per osteoclast between the studied groups. Both resorption pit number and percentage of resorption area were markedly increased after culture day 14 in cells from patients treated with TNFi as compared to baseline, reaching statistical significance at culture day 21 (p = 0.0469 for both resorption pit number and percentage of resorbed area; Fig 3B).

Gene expression by RT-qPCR was performed for OC genes that are known to be important during its differentiation and activity. All genes, except CTSK, were significantly lower at culture day 1 in patients after TNFi treatment when compared to healthy donors (CSF1R p = 0.0186; RANK p = 0.0095; NFATc1 p = 0.0015; ATP6V0D2 p = 0.0004; Fig 4). At culture day 1 RANK expression in patients at baseline was significantly lower than in healthy donors (p = 0.0268; Fig 4). There were no differences between patients and controls or between baseline and post TNFi treatment patients, except for ATP6V0D2 expression at culture day 21 in patients at baseline, which was significantly lower than in healthy donors (p = 0.039; Fig 4) and than in patients after TNFi (p = 0.0234). After correcting for multiple comparisons, expression of RANK, NFATc1 and ATP6V0D2, in culture day 1, from patients after TNFi remained significantly lower than in healthy donors.

No differences were found in any of the studied parameters when comparing presence or absence of HLA-B27, presence or absence of peripheral involvement or comparing monoclonal antibodies (Adalimumab, Infliximab or Golimumab) to the fusion protein Etanercept (data not shown).

Discussion

We have shown that in TNFi treated AS patients, the pro-inflammatory and pro-osteoclastogenic systemic stimuli were decreased due to reduced $RANKL^+$ circulating lymphocytes (B and T cells) and reduced levels of IL-17A and IL-23. Accordingly, OC specific gene expression was reduced in circulating precursors after TNFi exposure. However, when these precursors from TNFi treated AS patients were cultured in OC differentiating conditions, reproducing the bone

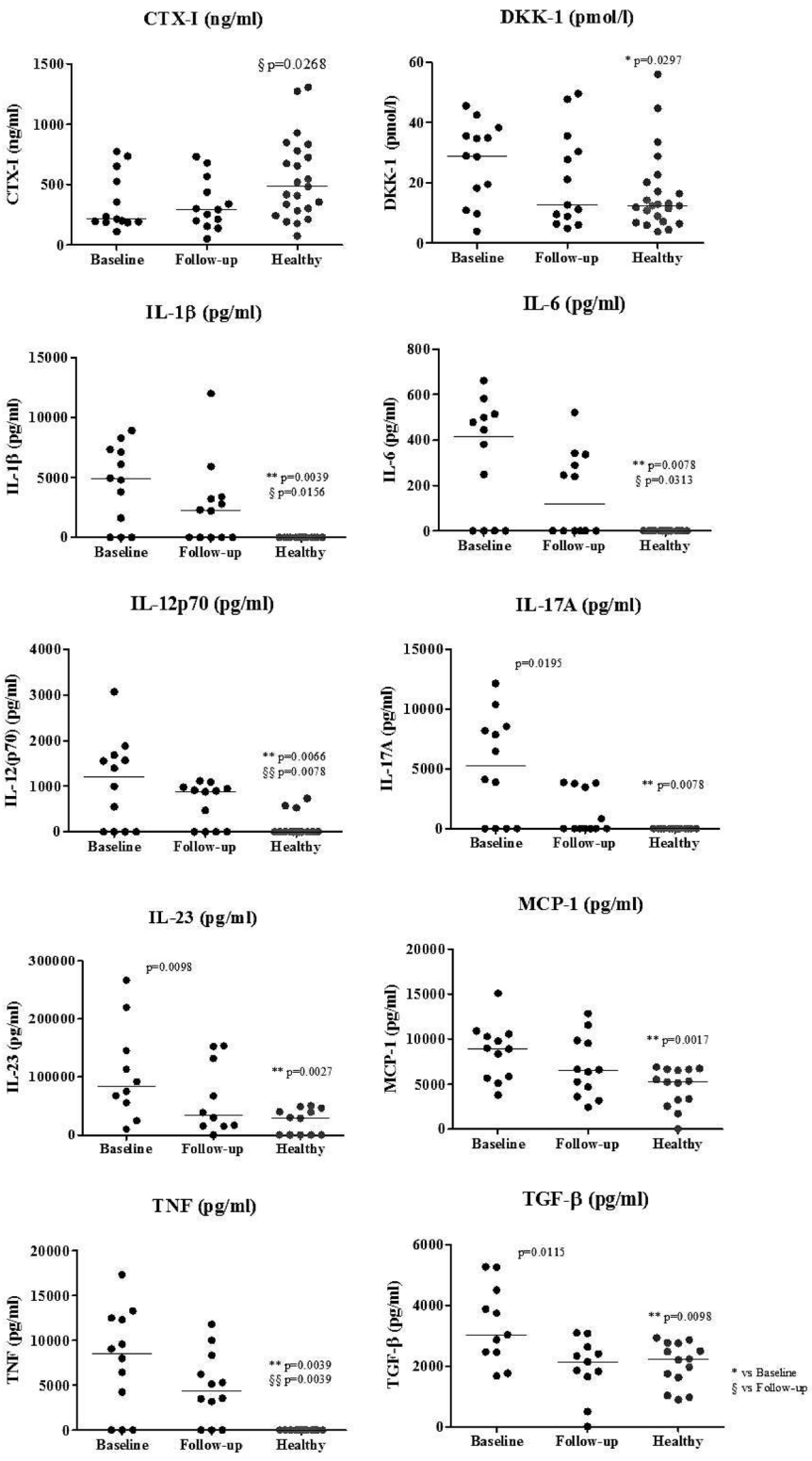

Fig 2. Serum levels of bone turnover markers, bone metabolism proteins and cytokines. CTX-I levels are decreased in patients at 6 months follow-up when compared to healthy donors (p = 0.0268). DKK-1, IL-1 β, IL-6, IL-17A, IL-12p70, IL-23, TNF, MCP-1 and TGF-β are increased in patients at baseline when compared to healthy donors. After 6 months of therapy, follow-up patients had decreased levels of IL-17A, IL-23 and TGF-β when compared to their baseline. We have also observed that after therapy the levels of IL-1 β,

IL-6, IL-12p70 and TNF were still significantly higher than healthy donors levels'. Each dot represents a sample. Line represents median. * vs Baseline, § vs Follow-up. DKK—dickkopf-related protein, CTX—carboxy-terminal collagen crosslinks, IL—interleukin, TNF—tumor necrosis factor, MCP—monocyte chemmotractant protein, TGF—transforming growth factor.

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microenvironment, their response to osteoclastogenic stimuli and activity was increased in comparison to baseline behavior.

One of the limitations of our study is the sample size and these results should be confirmed in a larger population. Another limitation is the use of circulating precursors. Bone local samples would have been preferred to use, however surgery to this bone areas of interest are rare and in addition it would be most difficult to obtain healthy controls for these samples. Our strategy was to address the question of circulating precursors being an important source of osteoclasts in active disease and the fate of their osteoclast differentiation ability after exposure to TNFi.

Our study found no correlation between monocyte phenotype, osteoclast activity or gene expression and treatment duration. Although there have been suggestions that longer therapy duration increases bone mineral density in AS patients [20] these studies have been performed in longer time courses than our study. We also found no differences between the use of monoclonal antibodies (Adalimumab, Golimumab and Infliximab) and the recombinant protein Etanercept. Most of the literature does not compare different TNFi [21,22] and thus more studies are needed to address if differences might exist at the level of radiographic progression.

Recent studies and meta-analysis have shown that TNFi treatment in AS patients is associated with increased lumbar and hip BMD [23]. In addition, an increased likelihood of developing new bone following resolution of inflammation after TNFi therapy has been suggested. Accordingly, radiographic progression was associated with decreased systemic inflammation and, on the contrary, radiographic nonprogression was associated with persistent inflammation, as assessed by IL-6 and CRP levels and MRI, supporting a link between the resolution of inflammation and new bone formation in AS patients during TNFi therapy [24,25].

It has been previously reported that neutrophils are more active in AS patients [26] but no differences in total B or T cell numbers were reported [27]. In our study no differences were found in the frequency of granulocytes and T and B cells between any of the studied groups. However, $RANKL^+$ neutrophils count was increased in patients at baseline and TNFi treatment reduced the number of $RANKL^+$ T and B cells. Previous studies have shown that T lymphocytes from AS patients have higher expression of RANKL than healthy donors [28], but to our knowledge a comparative study of RANKL expression in AS patients before and after TNFi was never been published.

In previous studies AS patients under NSAID therapy have been showed to have increased circulating number of classical monocytes and decreased non-classical monocytes when compared to healthy donors [29]. However, in our cohort, no differences were detected in any of the circulating monocytes subpopulations. In the intermediate monocyte subpopulation, patients exposed to TNFi had decreased CCR2 expression. CCR2 is a chemokine receptor that binds MCP-1 and promotes osteoclast precursors fusion and maturation [30]; its reduction after treatment is in accordance with the reduced gene expression of specific osteoclast genes observed in cells from patients after TNFi treatment and with its previously described role in osteoclastogenesis [30,31]. On the other hand, CD62L (L-selectin) was higher in AS patients after TNFi therapy in all three monocyte subpopulations when compared to healthy donors suggesting that adhesion is increased in these cells after exposure to TNFi. We speculate that our observation of high L-selectin (CD62L) expression in circulating monocytes

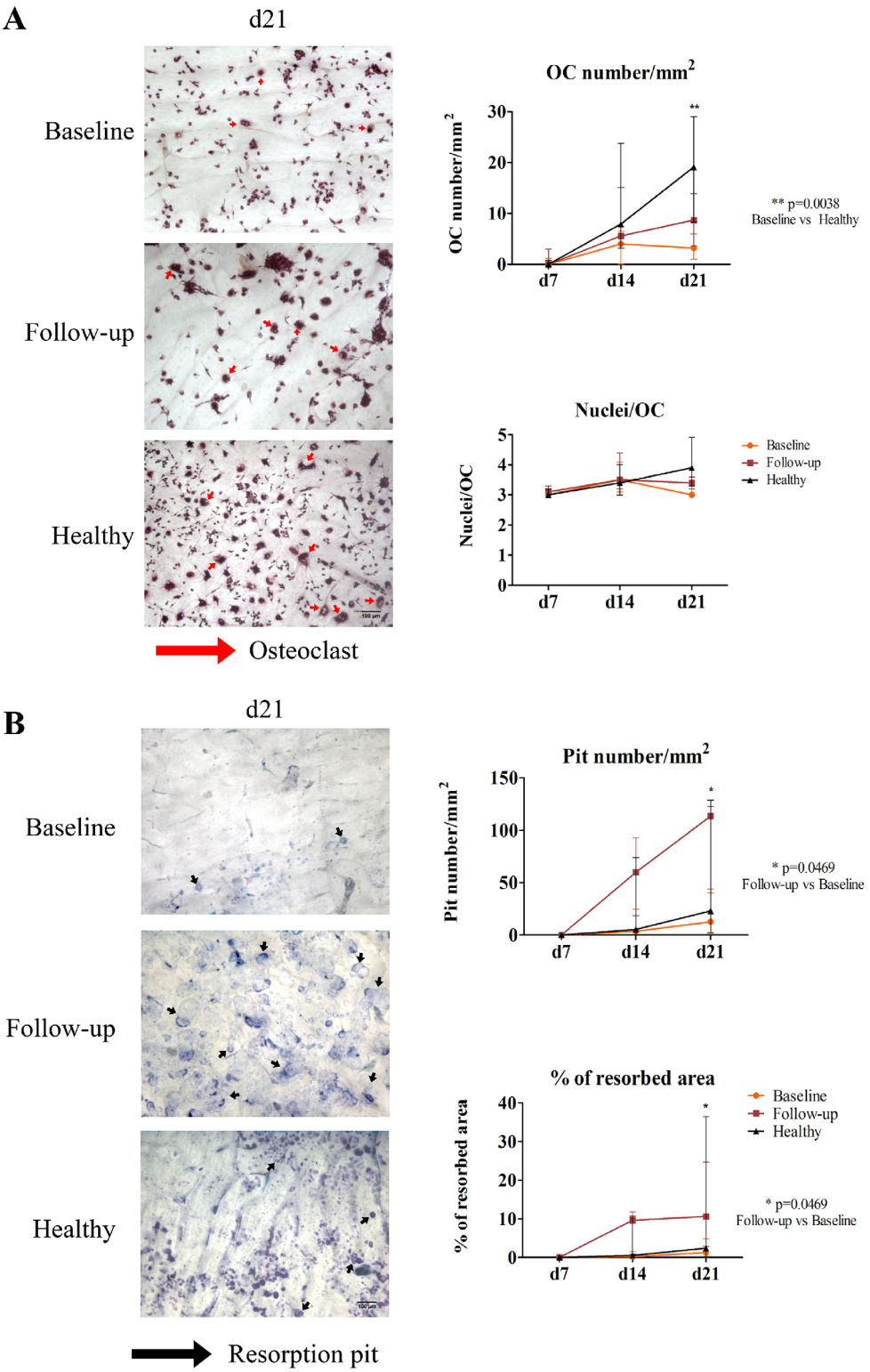

Fig 3. Osteoclast number is reduced in baseline patients, but bone resorption activity is increased after TNF-blocker exposure. A) Representative images of culture day 21 of cells stimulated with M-CSF, RANKL, dexamethasone and TGF-β and stained for TRAP. OC number increased throughout time and at culture day 21 baseline patients have significantly less osteoclasts than healthy donors (p = 0.0038). No differences were found in the number of nuclei per OC in any studied time of culture. B) Representative

images of pit assay at culture day 21. Patients at follow-up had significantly higher number of pits and resorption area at culture day 21 when compared to their baseline (p = 0.0469 for both resorption pit number and percentage of resorbed area). Dots represent median counts for each group at each time-point and bars represent interquartile range [10–90]. d—day; OC—osteoclast; Scale bars 100μm, red arrows—osteoclasts, black arrows—resorption pits.

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subpopulations after TNFi treatment may be related to increased adhesion of OC precursors to bone slices and subsequent cell activation. It was previously described in rats that the binding of L-selectin to some of its ligands (namely GlyCAM-1) increases integrin binding (β2 and also α4) [32–34]. Therefore, binding of L-selectin to ligands on the bone slice might increase αvβ3 integrin binding leading to increased OC differentiation [35]. We suggest that when osteoclast precursors attach to bone slices through integrins and L-selectin (CD62L), signaling pathways are activated and the expression of OC differentiation genes is induced. It has been previously shown that attenuation of the integrin $\alpha_V\beta_3$ pathway leads to inhibition of OC differentiation and that there is a crosstalk between integrin β_3 and M-CSF/c-fms pathways [36]. We further suggest that cell adhesion to bone by integrins plays an important role in OC differentiation, but additional studies are required to determinate how integrins are able, *per se*, of inducing OC differentiation.

As previously reported, serum levels of IL-1β, IL-6, IL-12p70, IL-17A, IL-23, MCP-1, TGF-β and TNF were significantly higher in AS patients with active disease when compared to healthy subjects [37,38]. In addition, IL-17A, IL-23 and TGF-β were significantly decreased in AS patients after TNFi therapy. IL-17A and IL-23 are well known for their role in the pathogenesis of inflammatory disorders, such as AS [39], and TGF-β is an important cytokine for bone formation [40]. In accordance with our study, Limón-Camacho *et al* found that serum levels of IL-17A were significantly elevated in AS patients with active disease, when compared to patients receiving TNFi; the same findings were observed for IL-6, IL-12, and TNF [37]. Moreover, we observed that the levels of most cytokines present in the serum

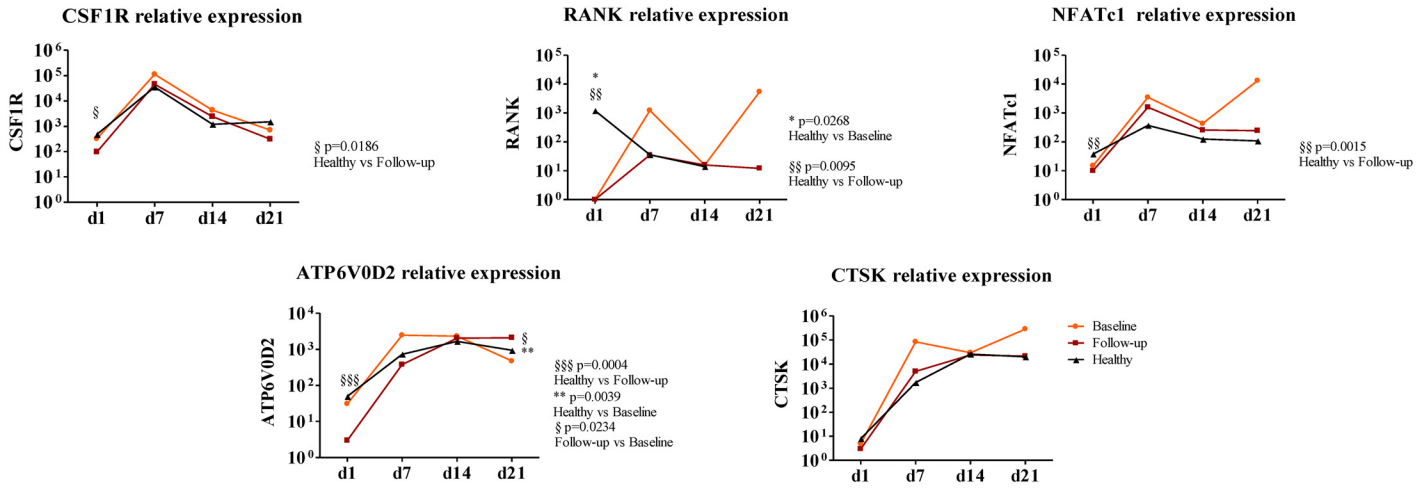

Fig 4. Gene expression profile of stimulated cells in culture for 21 days. All genes except CTSK are significantly decreased at day 1 in patients at follow-up when compared to healthy donors. At day 1 RANK expression in patients at baseline is also significantly reduced when compared to healthy donors (p = 0.0268). We found no differences between groups throughout the culture time except for Atp6v0d2 expression at day 21 in patients at baseline which is significantly reduced when compared both to healthy donors (p = 0.039) and patients at follow-up (p = 0.0234). Relative gene expression shown in Log scale. Dots in graphs represent median gene expression for each group at each time-point. d—day; CSF1R—gene encoding macrophage-colony stimulating factor (c-fms), RANK—gene encoding for receptor activator of nuclear factor-κβ, NFATc1—gene encoding for nuclear factor of activated T-cells, Atp6v0d2—gene encoding ATPase, H^+ transporting, lysosomal V0 subunit D2, CTSK—gene encoding cathepsin K. p<0.05 is considered significant.

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of AS patients normalized to the healthy donors levels after 6 months of TNFi therapy, indicating a reduction in the inflammatory environment induced by TNF blockade. Concordantly, patients under TNFi had reduced levels of CTX-I, suggesting a decrease in osteoclast activity. However, no differences in the levels of sRANKL, OPG and their ratio, before and after TNFi were found. Previous studies have shown discrepancies in sRANKL and OPG levels in AS patients. While sRANKL and OPG have been found increased in AS patients with active disease [41], in patients with mild to active disease sRANKL/OPG was lower than in healthy controls [38,42]. These latter studies found that after TNFi, sRANKL/OPG ratio was increased due to a decrease in OPG; however, none of the studies used paired patients samples.

SOST and DKK-1 have been implicated in the pathophysiology of AS, either by their reduced expression or by their functional de-regulation [43]. In our study, DKK-1 serum levels were higher in patients at baseline when compared to healthy controls; however, no significant differences in SOST or DKK-1 serum levels after treatment were found. Taylan *et al* reported that patients under TNFi presented higher DKK-1 levels compared to patients under conventional therapy (NSAIDs and/or DMARDs) [38]. More recently, Ustun *et al* found that TNFi did not affect DKK-1 and SOST levels [44]. However, several other studies suggested that DKK-1 levels decrease after TNFi and that there is no change in SOST circulating levels [43].

To understand the effect of TNFi in osteoclast differentiation and function we isolated PBMCs from AS patients before and after TNFi and cultured them *in vitro* over bone slices. OC formation continuously increased up to day 21. Both controls and patients (before and after TNFi treatment) exhibited the same pattern of increase in the number of resorption pits and the percentage of resorbed area over time, although at day 14 there was a marked increase in resorption in the TNFi treated patients that reached statistical significance at day 21. After TNFi treatment no differences in pit size were found suggesting that OC mobility is not affected by the disease or therapy and we found no differences in the number of nuclei/osteoclast, which has been suggested to correlate with osteoclast activity [45–47].

There is evidence that TNF contributes to expression of specific OC proteins and that it directly activates OC differentiation through cross activation of the NF-κB pathway or c-Jun N-terminal kinase (JNK) signaling cascade [48]. In our study, all genes, with the exception of cathepsin K, were downregulated after TNFi treatment. However, this reduced gene expression did not impair OC differentiation when the PBMCs were cultured under OC differentiating conditions. At later time points, culture days 14 and 21, there was a significant increase in ATP6V0D2 expression in healthy and TNFi treated patients, which might be related to our observation of increased bone resorption after TNFi.

In summary, in AS patients TNFi treatment reduces systemic pro osteoclastogenic stimuli. However, TNFi effect on OC precursors from AS patients increases their response to osteoclastogenic stimuli and their activity in bone pro inflammatory microenvironment. This is in disagreement with the apparent increase in osteoproliferation in AS patients treated with TNFi [24,25]. However, patients treated early in the course of the disease appear to escape this effect [49]. We hypothesize that early TNFi treated patients have an early normalization of bone resorption by TNFi, thus preventing osteoproliferation.

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Author Contributions

Conceived and designed the experiments: IPP JC JEF HC MA. Performed the experiments: IPP RR JC. Analyzed the data: IPP RR JC RC CP ES. Contributed reagents/materials/analysis tools: IPP RR RC CP ES HC. Wrote the paper: IPP RR JC RC CP ES HC MA JEF.

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