Aus der Medizinischen Klinik mit Schwerpunkt Rheumatologie und Klinische Immunologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

The role of endogenous glucocorticoid signaling in osteoblasts on inflammation and bone destruction by autoimmune arthritis

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Edgar Wiebe

aus Leningrad

Datum der Promotion: 17.09.2021

Für meine Eltern und Großeltern

Vorwort

Teilergebnisse der vorliegenden Arbeit wurden veröffentlicht in:

C. M. Spies, E. Wiebe, J. Tu, A. Li, T. Gaber, D. Huscher, M. J. Seibel, H. Zhou, F. Buttgereit. Acute murine antigen-induced arthritis is not affected by disruption of osteoblastic glucocorticoid signaling. BMC Musculoskelet Disord 2014 Feb 3;15:31. PMID: 24491163

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

11β-HSD1/2	11β-hydroxysteroid dehydrogenase type 1/2
ACPA	antibodies to citrullinated protein antigens
ACTH	adrenocorticotropic hormone
AIA	antigen-induced arthritis
AP-1	activator protein-1
basic-FGF	basic fibroblast growth factor
BMD	bone mineral density
BV/TV	bone volume/tissue volume
C5aR	complement component 5a receptor
CAIA	collagen antibody-induced arthritis
cDNA	complementary deoxyribonucleic acid
CFA	freund's complete adjuvant
CGB	corticosteroid-binding globulin
cGCR	cytosolic glucocorticoid receptor
Col2.3	2.3-kb collagen type iα1 promotor
CRH	corticotropin-releasing hormone
CRP	c-reactive protein
CT	computed tomography
CTR	control(s)
DNA	deoxyribonucleic acid
DKK1	dickkopf-related protein 1
EDTA	ethylene diamine tetraacetic acid
FcγR	Fc-gamma receptor
G-CSF	granulocyte colony-stimulating factor
G6PI	glucose-6-phosphate-isomerase
GC	glucocorticoid(s)
GM-CSF	granulocyte macrophage colony-stimulating factor
GCR	glucocorticoid receptor
GRE	glucocorticoid receptor element
H&E	haematoxylin and eosin
HPA	hypothalamic-pituitary-adrenal
IC	immune complex
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IL	interleukin
iκB	inhibitor of nuclear factor κb
KC	keratinocyte-derived cytokine
LIF	leukemia inhibitory factor
M-CSF	macrophage colony-stimulating factor
MIP	macrophage inflammatory protein
mBSA	methylated bovine serum albumine

MCP-1	monocyte chemotactic protein-1
mGCR	membrane-bound glucocorticoid receptor
MHC	major histocompatibility complex
micro-CT	microfocal-computed tomography
MIF	macrophage migration inhibitory factor
MIG	monokine-induced by interferon-gamma
MIP-1α	macrophage inflammatory protein 1α
MKP1	mitogen-activated kinase phosphatase 1
MMP	matrix metalloproteinases
MCR	mineralocorticoid receptor
N.Oc/BS	osteoclast number/bone surface
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κB
NF-AT	nuclear factor of activated T cells
NK	natural killer T cells
NOc/BS	number of osteoclasts/bone surface
NOD	non-diabetic obesity
Ob.S/BS	osteoblast surface/bone surface
Oc.S/BS	osteoclast surface/bone surface
OPG	osteoprotegerin
PBS	phosphate-buffered saline
PFA	paraformaldehyde
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa-b ligand
RANTES	regulated upon activation normal t cell expressed and secreted
RF	rheumatoid factor
RNA	ribonucleic acid
ROI	region of interest
SD	standard deviation
SEM	standard error of the mean
SSWAHS	Sydney south west area health services
Tb.N	trabecular number
Tb.Sp	trabecular separation
Tb.Th	trabecular thickness
TCR	T cell receptor
tg	transgenic
TGF	transforming growth factor
TNF-α	tumor necrosis factor α
TRAP	tartrate-resistant acid phosphatase
u-PA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor

ABSTRACT (GERMAN)

Einleitung

Die Rolle endogener Glucocorticoide (GC) in der Modulierung von Immunprozessen bei der rheumatoiden Arthritis (RA) ist noch unverstanden. Buttgereit et al. konnten zeigten, dass die Unterbrechung des GC-Signalings in Osteoblasten zu einer deutlichen Abschwächung der Entzündungsaktivität und zur Erhaltung der Knochenstruktur im Mausmodell der K/BxN Serum-induzierten Arthritis führt – ein RA-Modell, das T-Zell-*unabhängig* ist und vor allem durch das angeborene Immunsystem geprägt wird.

Zielsetzung

Um zu bestimmen, ob die Modulation der inflammatorischen Antwort durch Osteoblasten auch T-Zellen involviert, untersuchten wir die Effekte der Unterbrechung des GC-Signalings in Osteoblasten in dem T-Zell-*abhängigen* Modell der antigen-induzierten Arthritis (AIA). Um längerfristige Effekte dieser Unterbrechung vor allem auf Knochenerosionen und Knochenumsatz zu untersuchen, hielten wir eine chronische Entzündung in Langzeitmodellen der AIA sowie der K/BxN-Arthritis aufrecht.

Methodik

Die Wirkung endogener GC in Osteoblasten wurde durch die transgene Überexpression des GC-inaktivierenden Enzyms 11β-Hydroxysteroid-Dehydrogenase Typ 2 (11β-HSD2) unter Kontrolle eines Osteoblasten-spezifischen 2.3-kb Kollagen-Typ-I-Promotors gehemmt. Die AIA wurde bei transgenen (tg) und Wild-Typ (WT) Mäusen durch intra-artikuläre Injektionen von methyliertem bovinen Serumalbumin (mBSA) ausgelöst und in einem Langzeitmodell aufrechterhalten.

Für das Langzeitmodell der K/BxN Serum-induzierten Arthritis erhielten tg und WT Mäuse intraperitoneale Injektionen mit KRN-Serum, welche die Arthritis durch Booster-Injektionen an Tag 14 und 28 aufrechterhielten.

Das Ausmaß der Arthritis wurde mittels klinischem Score und Gelenksdurchmesser gemessen bis zum Endpunkt an Tag 14 und 28 (AIA), bzw. Tag 42 (K/BxN).

Die betroffenen Gelenke wurden histologisch und beim K/BxN Langzeitmodell zusätzlich mit Micro-CT untersucht. Systemische Effekte der Entzündung auf den Knochenstoffwechsel wurden mittels Histomorphometrie und Micro-CT der Tibia bewertet.

Ergebnisse

Bei der AIA war, auch im Langzeitmodell, kein signifikanter Unterschied zwischen tg und WT Mäusen in Hinblick auf das Ausmaß der Arthritis zu erkennen.

Beim Langzeitmodell der K/BxN Serum-induzierten Arthritis zeigten tg Mäuse ebenfalls keine statistisch signifikanten Unterschiede in der Ausprägung der Arthritis verglichen mit WT Mäusen. Dies gilt auch für Veränderungen der Knochenstruktur in der Micro-CT Analyse.

Trotz des langen Zeitraums zeigten sich in allen Langzeitmodellen keine eindeutigen Einflüsse der Entzündung auf den systemischen Knochenstoffwechsel.

Schlussfolgerung

Im Gegensatz zur K/BxN Serum-induzierten Arthritis wird das AIA Modell nicht durch die Unterbrechung des osteoblastären GC-Signalings beeinflusst. Dies deutet darauf hin, dass die GC-abhängige Modulierung der inflammatorischen Prozesse durch Osteoblasten T-Zell-**unabhängig** ist. Zudem scheint dieser modulierende Einfluss von Osteoblasten in einem chronischen Umfeld weniger relevant zu werden. Dies grenzt die Bedeutung von osteoblastärem GC-Signaling in entzündlichen Gelenkprozessen auf (sub-) akute inflammatorische Phasen ein.

ABSTRACT (ENGLISH)

Background

The role of endogenous glucocorticoids (GC) in the modulation of immune responses in rheumatoid arthritis (RA) remains unclear. Previously, Buttgereit et al. showed that disruption of GC signaling in osteoblasts results in a marked attenuation of arthritis and the preservation of bone structure in the K/BxN serum-induced arthritis model – a model driven decisively by players of the innate immune system.

Objectives

In order to determine whether GC-dependent modulation of the inflammatory response by osteoblasts involves T cells, we studied the effects of disrupted osteoblastic GC signaling in the T cell-dependent model of antigen-induced arthritis (AIA). To investigate the impact on bone erosion and turnover in a chronic inflammatory setting, we prolonged arthritis in long-term experiments using the AIA as well as the K/BxN serum-transfer models.

Methods

Intracellular GC signaling in osteoblasts was disrupted by transgenic overexpression of 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) under the control of an osteoblast-specific 2.3kb-type I collagen promoter. AIA was induced in transgenic mice and their wild-type (WT) littermates by intra-articular injection of methylated BSA into one knee joint and prolonged in a long-term model.

In the K/BxN serum-transfer model, arthritis was induced and prolonged by subcutaneous injections of K/BxN serum on days 14 and 28.

The severity of arthritis was assessed by clinical scoring and joint size measurements until the respective endpoints at days 14, 28 (AIA) and 42 (K/BxN). Joints were assessed histologically, and for the K/BxN model additionally by micro-CT. Systemic effects of inflammation on bone metabolism were quantified by histomorphometry and micro-CT of the tibia.

Results

In the AIA model, no significant difference between tg and WT mice, even after repeated flares, was observed for the clinical course and histological indices of inflammation, cartilage damage and bone erosions.

In the long-term K/BxN serum-transfer model, arthritis severity was not significantly different in tg compared to WT mice. This was also corroborated in histological indices and erosions assessed by micro-CT.

In all experiments, inflammation did not show a net effect on systemic bone turnover.

Conclusions

In contrast to K/BxN serum-induced arthritis, murine AIA is not affected by a disruption of GC signaling in osteoblasts. This suggests that the GC-dependent modulation of immune-mediated inflammatory arthritis by osteoblasts in mice is T cell independent. This modulating effect seems to abate in a chronic inflammatory setting of K/BxN arthritis, narrowing the role of endogenous GC signaling by osteoblasts down to (sub-) acute inflammatory processes.

1 INTRODUCTION

1.1 **Rheumatoid arthritis**

1.1.1 Aetiopathogenesis – the role of the innate and adaptive immune system

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial inflammation followed by cartilage degradation and bone destruction. It affects approximately 1% of the worldwide population and is associated with a high degree of disability, morbidity and increased mortality (1). The underlying mechanisms of the disease comprise three main features: autoimmunity, chronic inflammation and ultimately, joint destruction (2). The overarching principle of autoimmunity is represented by the concept of immune dysregulation (3), with both the *innate* and *adaptive* immune system being crucial players in RA pathogenesis.

The innate immune system comprises humoral as well as cellular immune responses (4) and contributes, due to its inappropriate activation, to the autoreactive state in RA and thereby to the onset and progression of chronic inflammation (5). Being part of the humoral response, the activation of the complement system has been identified as an important contributor to RA (6) – in both the initiation and persistence of the disease involving pathologic citrullinated proteins (7-9), and also in the associated synovial inflammation (10, 11) and cartilage damage (12, 13).

Cells of the innate immune system also contribute to the pathogenesis of RA (5): Neutrophils, natural killer cells, monocytes/macrophages, innate lymphoid cells and dendritic cells exert their function as phagocytes, pro-inflammatory cytokine producers (14), and/or antigen-presenting cells (15). This not only causes tissue damage, but additionally directs the activation of the *adaptive* immune system (4), thereby contributing to the perpetuation of the disease (15).

The adaptive immune system is intrinsically tied to the concept of autoimmunity. In RA, autoimmunity is evidenced by the presence of autoantibodies such as anti-citrullinated protein antibodies (ACPAs) and IgG antibodies (rheumatoid factor, RF) (3, 16-19). B and T cells, as key players of the adaptive immune system, are critical promotors of autoimmunity by sustaining antibody production and perpetuating a complex cytokine network that mediates chronic inflammation (20-23).

Consequently, a synergistic interplay between the innate and adaptive immune system via auto- and paracrine cytokine networks (1) evolves. This represents the pathophysiological basis for the underlying chronic inflammation of the synovial milieu. At an anatomical level of joint pathology, chronic inflammation leads to synovial hyperplasia (with infiltration of both innate and adaptive immune cells) (24). This, in the long run, results in pannus formation (25), which eventually cascades into the destructive phase of the disease with cartilage degradation and bone destruction (26, 27) - with bone loss representing an epitome of the disease (28), associated with a high disease burden (29).

1.1.2 The effect of inflammation on bone in RA – focal and systemic bone loss

The microenvironment with abounding chronic inflammation leads to structural bone damage inside the joints in the form of bone erosions (30, 31), i.e., focal bone loss. In addition, microarchitectural changes of trabecular and cortical bone occur, referred to as 'periarticular' osteopenia (32). Apart from these pathognomonic features of RA at the joint level, patients with RA are also at an increased risk for *systemic* bone loss, leading to osteoporosis and subsequently, an increased risk for fragility fractures (33-36). Both local bone erosions and systemic bone loss are attributed to chronic inflammation and its underlying processes (37), where inflammation leads to an imbalance of bone remodeling with increased bone resorption (enhanced osteoclast differentiation) and impaired bone formation (inhibited osteoblast function). This tight interplay between bone cells and immune cells has long been recognized, giving rise to the field of osteoimmunology since the early 2000s.

To go into more detail, a plethora of pro-inflammatory cytokines exerts pro-osteoclastogenic effects (38, 39). For example, macrophage colony-stimulating factor 1 (M-CSF), tumor necrosis factor (TNF)-α, Interleukin (IL)-6, IL-1, and leukemia inhibitory factor (LIF) either directly or indirectly enhance osteoclast activity and osteoclastogenesis (40) via the receptor activator of nuclear factor κB (NF- κB) ligand (RANKL) (41-43). B and T cells, through secretion of cytokines as well as through direct stimulatory effects on osteoclasts via RANKL (44), contribute to the process of bone remodeling in RA; B cells, by producing ACPAs and RF, are considered a main driver of bone loss. The presence of autoantibodies induces osteoclastogenesis (45, 46) and

is associated with a more severe clinical course of arthritis in terms of bone erosions as well as systemic bone loss (32). The important role of T cells in bone homeostasis is exemplified by the fact that T cells (especially Th1, Th2, Th17 cells) are the main secretors of cytokines such as IL-17 (47) and IL-15 (48) that drive osteoclastogenesis. In addition, they also engage in direct crosstalk with osteoblasts: T cells amplify the osteoblastic response to PTH via a pathway that critically depends on Wnt signaling (49). In fact, B and T cell-deficient mice were shown to have an osteoporotic phenotype (50). In contrast, however, mice deficient in T cells seem to be protected from postmenopausal bone loss (re-administration of T cells from wild-type mice led to bone loss) (51).

1.1.3 Osteoblasts as immunocompetent cells

Apart from the negative stimuli of inflammation on bone cells, bone cells themselves share many common progenitors and receptors with cells of the immune system (52). Osteoblasts and osteoclasts themselves are considered immunocompetent cells. Osteoblasts directly regulate osteoclastogenesis via RANKL and osteoprotegerin (OPG), where RANKL binds to the RANK receptor on the membrane of osteoclast-precursors (monocytes from the mesenchymal progenitors), while OPG, a decoy receptor of RANKL, prevents RANKL/RANK binding and thereby osteoclastogenesis (53). Furthermore, osteoblasts have also been shown to express complement factor C3 and C5 and secrete IL-6 (54) via a complement-dependent pathway (55).

In order to prevent bone loss and joint destruction mediated by autoimmune processes in RA, many treatments have evolved over the past few decades and have been shown to be efficacious by targeting cytokines and/or intracellular pathways (14). Glucocorticoids (GCs) were one of the first potent anti-inflammatory and immunosuppressive drugs, and even today they remain a central element in RA treatment (56). GCs are, in fact, of special interest in terms of arthritis and bone. While on the one hand, GCs are indispensable in the treatment of autoimmune diseases with underlying bone destruction, on the other, GCs may themselves exert a negative impact on bone.

1.2 **Glucocorticoids**

1.2.1 Exogenous glucocorticoids and their effects on inflammation and bone

The first discovery of the potent anti-inflammatory and immunomodulatory (57) properties of 'compound E' (later called 'cortisone') in 1948 by Hench and Kendall (58) was a ground-breaking one, marking the beginning of physicians' ability to treat autoimmune diseases (59). Ever since, GCs continue to be a cornerstone in the treatment of rheumatic diseases (60, 61).

In RA, GC treatment results (through pleiotropic effects on innate and adaptive immune cells) (62) in a reduction of synovial inflammation and a slowing of radiographic progression and joint damage (63-65) – just to name a few of its manifold effects.

However, prolonged and high dose GC treatment also results in adverse effects, especially with regard to the bone: treatment with GCs is associated with significant loss of bone density and structure, and thus an increased fracture risk (66, 67). High levels of GCs are considered to have direct effects of bone cells such as osteoblasts, osteocytes and osteoclasts, with the proposed mechanisms being impaired cellular proliferation, increased apoptosis, and changes in RANKL/OPG, Wnt/sclerostin expression (68).

High doses of GCs slow the proliferation of osteoblasts and seem to affect the differentiation from progenitors to other lineages such as adipocytes, as is exemplified by the observation that GC-treated RA patients show a higher rate of adipose tissue in the femoral neck (69). GCs stimulate expression of RANKL by osteoblasts and suppress their OPG expression. Also, GCs stimulate osteoblast apoptosis, suppress insulin-like growth factor (IGF)-1 expression (70) and hinder transforming growth factor (TGF)-β actions (71).

In addition, GCs also exert effects on osteocytes - they are known to induce osteocyte apoptosis in vivo and in vitro (72-74). Osteocytes participate in bone remodeling by secretion of RANKL and OPG and thus regulate osteoclastogenesis (75). GCs lead to a reduced osteocytic OPG expression which increases the impact of RANKL (76). At the same time they increase production of Dickkopf-related protein 1 (DKK1) (77) and sclerostin (78), which are potent Wnt signaling-inhibitors (79). This, in turn, negatively affects osteoblastogenesis.

While exogenous (i.e. therapeutic) GCs and their effects on inflammatory processes have been well characterized (as have their side-effects) (80), the contribution of endogenous GCs in autoimmune processes is much less understood. This is somewhat paradoxical, since endogenous GCs play an essential role in the physiological homeostasis of many organs and functions, including the regulation of physiologic immune responses (81).

1.2.2 Endogenous glucocorticoids

GCs are metabolic hormones endogenously produced in the zona fasciculata of the adrenal cortex as part of the hypothalamic-pituitary-adrenal (HPA) axis where GC production and levels are regulated by a negative feedback loop underlying a strict circadian rhythm (82-84). This mechanism involves a hierarchical system with adrenocorticotropic hormone (ACTH) from the pituitary gland as a stimulator of GC production and corticotropin-releasing hormone (CRH) from the hypothalamus as a stimulator of ACTH, while circulating GC levels affect secretion of both ACTH and CRH.

GCs (both endogenous and exogenous) exert their effects via genomic and non-genomic actions (85). For the genomic action, GCs pass through the lipophilic cell membrane into the cell and bind to a cytosolic GC receptor (cGCR). This complex then translocates into the cell nucleus and either directly binds via a GC response element (GRE) on DNA, or indirectly via transcription factors, further affecting the synthesis of cytokines by transactivation or transrepression (86), respectively. Transactivation (through binding of a *dimerized* GRE on DNA) leads to the increase of anti-inflammatory and anti-proliferative cytokines such as IL-10 or inhibitor of nuclear factor kB (ikB) (87). Transrepression refers to the modulation of pro-inflammatory cytokines through the binding of a *monomeric* GCR to transcription factors like NF-κB (88) or activator protein-1 (AP-1), thereby leading to an inhibition of these factors and thus, indirectly, to an inhibition of pro-inflammatory cytokine expression (89) such as nuclear factor of activated T cells (NF-AT), IL-1, IL-2, TNF and interferon (IFN)-γ (90, 91). The genomic action is considered to occur within 30 minutes of GC exposure.

For more rapid effects within seconds and minutes, non-genomic actions have been proposed (92). The pathways that are involved in the non-genomic mechanisms are multifold and are considered to be due to: a) non-specific interactions of GCs with cellular membranes (especially at high concentrations); b) effects mediated by the cGCR (inhibition of arachidonic acid); and c) due to effects that are exerted by membrane-bound GC-receptors (mGCRs) found on monocytes (93) and T cells (94).

Although the extent of GC effect is to a large degree related to its circulating levels, at a tissue and cellular level, the effect of GCs is regulated by several mechanisms, perhaps most relevantly by: a) the expression and sensitivity of GC-receptors (84); and b) the metabolism of GCs at a pre-receptor level by enzymes. Concerning the first, dimerization of the GR in T cells has been shown to be indispensable for anti-inflammatory effects (95). Concerning the latter, pre-receptor metabolism is of particular interest in the context of this study and shall be elucidated further.

1.3 **11β-hydroxysteroid dehydrogenases (11β-HSDs)**

At a pre-receptor level, 11β-HSD enzymes interconvert the active GCs cortisol and corticosterone with their inactive counterparts cortisone and dehydrocorticosterone, and thus retain the capacity to influence the differentiation and function of cells involved in inflammatory processes. Cortisol and corticosterone exert similar actions on the glucocorticoid receptor (GCR) as well as the mineralocorticoid receptor (MCR), with both having a 10-fold higher affinity for the MCR (96). In mineralocorticoid target tissues such as the kidney (97), and the salivary (98) and sweat glands (99), 11β-HSD type 2 (11β-HSD2) inactivates cortisol to cortisone and corticosterone to dehydrocorticosterone, which consequently lose their affinity to GCR and MCR. The reactivation of cortisol from cortisone is brought about by 11β-HSD1, which, in contrast to 11β-HSD2, is expressed at many tissues such as liver and bone (100).

1.3.1 The role of endogenous glucocorticoid signaling in arthritis

In the past few years, the understanding of the role of endogenous GCs in autoimmune arthritis has grown substantially. Starting with the observation that the HPA axis in patients with rheumatoid arthritis is deranged (expressed by a lost circadian rhythm of

cortisol secretion which correlates with inflammatory activity (101) and an impaired secretion upon CRH stimulation (102)), evidence has accumulated that endogenous GCs contribute fundamentally to the regulation of inflammation at a local as well as systemic level, essentially through GC metabolism at a pre-receptor level. Endogenous GC signaling affects immune cells (103), chondrocytes (104), and osteoblasts as well as osteocytes (105). Just recently, Fenton et al. demonstrated that global deletion of 11β-HSD1 in arthritic mice resulted in a resistance to GC treatment, while deletion of mesenchymal 11β-HSD1 did not (106). This suggests that systemically inactivated GCs require peripheral reactivation by 11β-HSD1 from sites of inflammation.

11β-HSD1 is known to be upregulated in the synovial tissue of fibroblasts and macrophages in patients with RA (107, 108), and has been shown to regulate local inflammation, synovitis and joint destruction (109, 110). Conversely, 11β-HSD2, as a counter-player, was expressed at a higher 11β-HSD2/11β-HSD1 ratio in the synovial fibroblasts (111) of patients with RA compared to patients with osteoarthritis, hinting at the possibility of an impaired capacity of synovial cells for local reactivation of cortisone in RA. Gene expression of 11β-HSD2 has been demonstrated to be overexpressed in synovial macrophages in RA patients (112). In addition, 11β-HSD2 expression has also been found at low levels in human osteoblasts (113) and, most prominently, in human osteosarcoma cell lines (114).

These findings herald a potential role of 11β-HSDs in the regulation of GC metabolism in bone cells.

1.3.2 The role of endogenous glucocorticoid signaling in bone

While 11β-HSD1 activity in osteoblasts increases with age and GC exposure (115), 11β-HSD2 expression in bone is only present during fetal development and declines afterwards (116). Through *transgenic* expression of 11β-HSD2 in osteoblasts and osteocytes, however, our understanding of the effects of endogenous GCs on osteoblasts has been tremendously enhanced. By targeting the 2.3Kb Collagen type I promoter, which is only expressed in mature osteoblasts and osteocytes, 11β-HSD2 can be exclusively *over*expressed - thereby abrogating completely endogenous GC signaling inside the osteoblast and osteocyte. This is reflected by the fact that transgenic mice are protected from the above-mentioned negative GC effects on

osteoblast-mediated bone metabolism (117, 118). Over-expression of 11β-HSD2 leads to a phenotype of slightly reduced bone density of the vertebrae, delayed ossification and reduced periosteal apposition of new bone (118, 119).

Zhou et al. have shown that in vivo, the differentiation of osteoblasts critically depends on endogenous glucocorticoids and their effects on Wnt signaling (120, 121). At first glance, this appears in sharp contrast to the negative effects of GCs on bone, especially considering that DKK-1, a Wnt-inhibitor expressed by osteoblasts, is induced by GCs resulting in a suppressed expression of OPG and osteoblastogenesis (122). Notably, the negative effects of GCs on osteoblasts seem to be related to the expression of a monomeric form of the GC receptor, which is mainly involved in transrepression mechanisms at a genomic level (123).

However, further research has prompted the hypothesis of a biphasic construct, with high doses of GCs exerting an anti-proliferative and catabolic effect on osteoblasts, while low GC doses yield a potential stimulatory and anabolic effect as a result of GC-dependent regulation of Wnt signaling (124).

Nevertheless, the role of endogenous GCs and their involvement in inflammatory diseases remains insufficiently understood. With endogenous GCs playing an essential role in osteoblastic differentiation, and rheumatoid arthritis representing a disease in which both endogenous GC signaling and osteoblasts are relevant players in underlying pathology, the question arises as to how endogenous GCs in osteoblasts contribute to the initiation and modulation of arthritis.

In order to study the pathways of inflammation that are involved in RA, several animal models have proven useful in understanding the underlying immunologic mechanisms that are not or cannot be readily studied in humans or patients. In recent times, many different mouse models have evolved that reflect many parallels to human RA, but they differ from each other by involving distinct immunological processes. This can be regarded as an advantage in terms of scrutinizing and modelling distinct pathways under defined research objectives.

1.4 **The antigen-induced arthritis model**

In 1977, Brackertz et al. showed that autoimmune arthritis can be induced in various strains of mice after immunization with methylated bovine serum albumin (mBSA) and reexposure of mBSA into the knee joint by intra-articular injection, inducing a chronic and antigen-specific monoarticular arthritis that shares many common features with human RA (125). Importantly, this model is dependent on T cells, since mice deficient in T cells only develop arthritis after the transfer of syngeneic T cells. Specifically, CD4+ T cells recruit neutrophils and macrophages, which leads to a hyperplasia of synovium, pannus formation, cartilage damage and bone erosions. CD8+ T cells are dispensable (126). Reduced severity of the disease has also been shown to correlate with a reduction of Th1 and Th17 responses (127). Baschant et al. found that the effect of glucocorticoids to mitigate Antigen-induced arthritis is based on dimerization of the GC-receptor in T cells (128). Cytokine analysis in this model revealed the involvement of established pro-inflammatory cytokines in RA like IL-1β, IL-6, IL-17, in both the synovial fluid as well as the serum at different time points (129). The role of complement is less clear, with contradicting results, whereby in one study, inhibition of complement by cobra venum factor (CVF) did not seem to alter the course of arthritis (130), while in another, administration of CVF reduced severity of AIA at any time point (131). Also, selective C5a receptor antagonists clearly reduce the severity of arthritis (132, 133), which has also recently been achieved by the introduction of DNA vector with anti-C5 neutralizing recombinant antibody (134).

AIA is considered to be a valid model for investigating synovitis and joint destruction, well suited for elucidating the pathogenesis of arthritis-induced bone loss (135). Interestingly, the AIA model was originally described as a chronic model, with synovitis persisting for up to 24 weeks and even up to two years in rabbits (136). This was considered to be due to antigen persistence inside the joint in the form of immune complexes (137) which resemble human RA, where immune complexes have been shown to persist inside the articular cartilage and synovium (138). In practice, however, inflammation subsides rather quickly after administration, which led to the approach of re-injecting mBSA intra-articularly, thus prolonging inflammation (139). The re-exposure of the antigen intra-articularly induces flare-up reactions, but it also bears the risk of iatrogenic damage of the joint by multiple knee joint puncture which could

confound the assessment of chronic joint damage. Thus, van de Putte et al. proposed a model in which the intravenous re-administration of the mBSA antigen after initial intra-articular injection leads to an acute flare-up of arthritis, solely confined to the affected joint without systemic effects on other joints (140), suggesting a preferential deposition of immune complexes inside the chronically inflamed synovium (141). Histologically, this flare-up involves predominantly granulocytes, while immunochemistry and cytokine expression inside the synovial fluid has been shown to involve IL-1 as a key cytokine during the flare-up reactions (142), as well as IL-6 which drives inflammation-induced osteoclastogenesis (126).
In summary, the AIA model represents a convenient model to study interventional effects where the adaptive immune system plays a key role. Its advantage also lies in the possibility of inducing flare-up reactions that mimic active chronic disease, as is the nature of RA.

Since no single mouse model exists that could fully reflect the entire extent and pathology of human RA, a plethora of further animal models has been developed to study different aspects of the disease. An important model, commonly used and extensively described, is represented by the K/BxN serum-transfer model.

1.5 **The K/BxN serum-transfer model**

The K/BxN serum-transfer model is a model where arthritis is induced by passive transfer of autoantibodies from K/BxN mice, in whom arthritis develops spontaneously. K/BxN was originally described by Kouskoff et al. in 1996, when, by a sheer act of serendipity, transgenic mice C57BL/6 coding for a rearranged T cell receptor was crossed with a non-obese diabetic (NOD) mouse strain (143). These K/BxN mice all developed severe and progressive arthritis with a phenotype closely resembling human RA - i.e., involving all joints, with the characteristic symmetric inflammation being based on an underlying autoreactivity as a hallmark of RA. Furthermore, Korganow et al. developed a transfer model, the K/BxN serum-induced arthritis model, which elicits a phenotypically similar form of symmetric arthritis in otherwise normal mouse recipients. This serum-transfer model has been shown to be T cell- and B cell-independent, as arthritis is induced by serum antibodies, even in recipients that are

devoid of lymphocytes (144). In contrast, the innate immune system is critically involved in the development of this form of antibody-induced arthritis. Immune complexes of arthritogenic anti-glucose-6-phosphate isomerase (GPI) autoantibodies attract and activate neutrophils and macrophages at the cartilage surface through Fc receptor binding (particularly FcγRIII) which is expressed on mast cells, neutrophils, macrophages and NK cells (145). The inflammatory cascade is accompanied by the release of IL-1 and TNF-α (146) and activation of complement factors from the initial part of the alternative complement pathway (147-151). Importantly, the alternative pathway of complement seems to be of particular importance. C5-deficient mice do not develop arthritis, and correspondingly, arthritic mice treated with anti-C5 monoclonal antibodies showed an effective mitigation of arthritis, while C4 deficiency does not alter the course of arthritis (147).

Bone destruction in the K/BxN model is brought about by the inflammatory mechanisms activating osteoclasts, with RANKL playing (as in human RA) a central role. RANKL-deficient mice which are exposed to K/BxN serum developed unhindered arthritis with severe paw swelling, yet had significantly reduced bone erosions in micro-CT and histopathologic analysis (152). This is especially relevant in the context of absent T cells.

The K/BxN serum transfer model stands out in regard to bone metabolism by featuring the formation of new bone at cortical surfaces around the anterior tibia and calcaneus as well as forefoot bones, a process that seems to be RANKL independent (144, 152). Nevertheless, the K/BxN serum transfer model is a very suitable model with many similarities to human RA (autoantibodies, symmetric arthritis, bone erosions) and allows for the investigation of downstream processes of inflammation and bone erosions. With regard to our aim to understand the role of endogenous GC signaling in osteoblasts on inflammation and bone destruction, this model seems particularly appropriate.

1.6 **Rationale of this study**

Hypothesizing that osteoblastic GC signaling might influence the inflammatory response in autoimmune arthritis, in 2009 Buttgereit et al. used the above-mentioned transgenic mouse model of 11β-HSD2 overexpression in osteoblasts and induced arthritis in mice by the K/BxN serum-transfer model. Much to their surprise, they found that specific disruption of GC signaling in osteoblasts significantly attenuated all aspects of inflammation in the K/BxN serum transfer model. During a period of 14 days, the clinical course as measured by ankle size and clinical score was significantly mediated after approximately 10 days, as was corroborated by histological indices of inflammation, cartilage degradation and bone erosion at day 14 (153). Also, tg mice were protected from arthritis-induced systemic bone loss as measured by micro-CT and histomorphometry. This pointed to the ability of osteoblasts to regulate inflammatory processes via a GC-dependent mechanism and to a potential role as potent mediators of inflammation.

The K/BxN serum-induced arthritis model is a T cell-independent model. However, osteoblasts are known to interact with T cells through osteoblastic proinflammatory cytokine secretion (which is GC-modulated), prompting us to now investigate the effects of disrupted GC signaling in the T cell-dependent AIA model.

Also, with regard to the chronicity of human RA and the effects of arthritis on bone being intricately related to disease duration, we also wanted to investigate the effects of long-term arthritis on bone where GC signaling is disrupted in osteoblasts and address the question of whether the modulatory effect of osteoblasts persists over a longer period. This is especially relevant when considering that the degree of erosions in the study by Buttgereit et al. was relatively low. Also, a duration of 14 days might not involve remodeling processes which are described to have a delayed onset. Animal models studying arthritis-induced osteoporosis often come short of evaluating the long-term exposure to inflammatory diseases as compared to humans. Patients with RA show systemic bone loss even before the clinical onset of arthritis, potentially mediated by autoantibodies such as ACPA or RF (154).

To summarize, the aim of these studies was to investigate:

Aim 1: whether the modulation of inflammatory responses by osteoblasts involves T cells. For this purpose, we induced arthritis by the AIA model which is characterized by the involvement of the adaptive immune response.

Aim 2: whether the modulating role of osteoblasts persists in a long-term setting and whether long-term arthritis models reflect important aspects of arthritis-induced bone loss. For this purpose we prolonged both the AIA and K/BxN serum-transfer models by inducing flare-up reactions, i.e., re-exposure to antigen and autoantibodies, respectively, in order to assess the effect on bone in a long-term setting.

2 METHODS

2.1 Transgenic mouse model

Col.2.3.-11β-HSD2 transgenic mice were generated as described originally by Sher and Woitge et al. (118) and were kindly provided as a gift by Dr. Barbara Kream (Department of Medicine, University Connecticut Health Center, Farmington, CT). In accordance with institutional animal welfare guidelines and according to an approved protocol by the Sydney Local Animal Welfare Committee, mice were maintained under specific pathogen-free conditions at the animal facilities of the ANZAC Research Institute. Mice had access to food and water ad libitum and were exposed to a 12-hour light/dark cycle.

2.2 Arthritis models

2.2.1 The antigen-induced arthritis (AIA) model

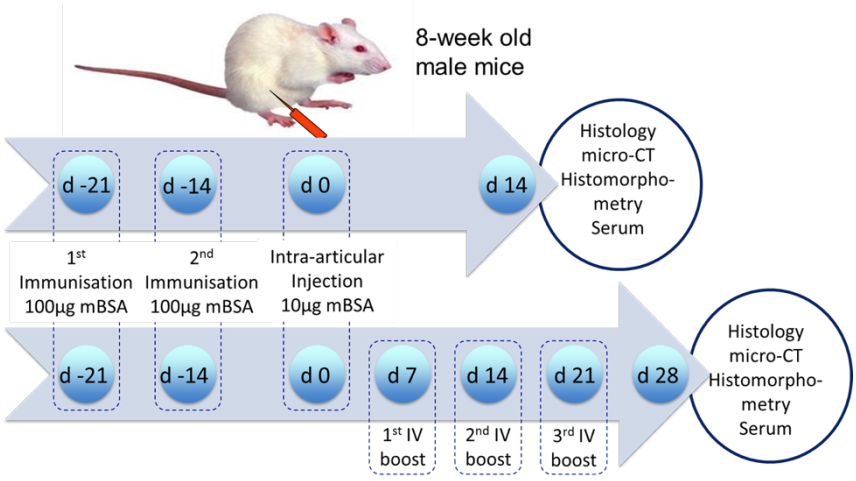

Figure 1: **Experimental design of the acute AIA (above) and prolonged AIA study (below)**

2.2.1.1 Immunization

Antigen-induced arthritis (AIA) in mice was induced according to established protocols (155, 156). As a model involving primarily the adaptive immune system, arthritis induction was based on an initial immunization. 100µg of methylated bovine serum albumin (mBSA) (Sigma, Castle Hill, Australia) was dissolved in 50µl of phosphate-buffered saline (PBS) and emulsified in 50µl of Freund's complete adjuvant (CFA) (Sigma, Castle Hill, Australia). On day -21, eight-week-old male Col2.3.-11β-HSD2 tg

mice and their WT littermates were immunized by subcutaneous injections of 50μg in each flank. A second injection of the same dose was given seven days later on day -14 by subcutaneous injection into the tail base (Figure 1). Control mice were also immunized with mBSA. Mice were randomly assigned to their respective groups matched for body weight and litter.

2.2.1.2 Induction of acute AIA

Induction of acute AIA was achieved by intra-articular injection of 10μg mBSA dissolved in 5μl sterile PBS on day 0 in tg mice (n = 17) and their WT littermates (n = 17), following brief inhalation anesthesia with isoflurane. Using a Hamilton syringe (Sigma, Castle Hill, Australia) and a $25^{1/2}$ gauge needle, the patellar ligament of the right knee was punctured until reaching intra-articular space, with the knee joint positioned at an angle of least tension on the patellar ligament. Control tg and WT mice received 5μl of sterile PBS. Body weight and joint swelling were assessed daily or every other day, from induction on day 0 until day 14. Knee joint swelling was measured using a Vernier caliper. Maximum medial-to-lateral diameter was defined at the widest point of each knee joint. For control purposes, the contralateral knee joint was also measured in order to monitor potential but unlikely symmetric effects after induction of acute AIA. Knee joint swelling was expressed as the absolute difference to baseline knee-joint diameter at day 0. On day 14, mice were euthanized for tissue and blood collection. A limited number of samples (n = 5 for each group) of synovial tissue from the affected knee joint, bone and bone marrow was preserved for future gene- and protein expression analyses. Samples were sent after storage at -80°C to our laboratory in Berlin, where RNA was extracted and recoded to cDNA.
For the remaining samples, histological, micro-CT, histomorphometric and serum cytokine analyses were performed as described below.

2.2.1.3 Induction of prolonged arthritis

In order to investigate the long-term effects of arthritis on bone and the potential role of endogenous glucocorticoids, arthritis was prolonged using an established model (155, 156). In order to avoid potential mechanical damage by repeated intra-articular injections of mBSA, we induced flares of arthritis by intravenous re-injections of mBSA,

as was described by Lens et al. (155). After induction of acute AIA in tg and their WT littermates, arthritic mice received repeated intravenous injections of 300μg mBSA dissolved in 200μl sterile PBS on days 7, 14 and 21, while tg and WT controls received 200μl sterile PBS only (n = 10 per arthritic group, n = 9 per control group) at the same time points. Body weight and clinical assessment were performed continuously, as described above. On day 28 after initial arthritis induction, mice were euthanized by cervical dislocation under anesthesia after blood collection. For the long-term experiment, eight mice had to be excluded for following reasons: five AIA mice had anaphylactic reactions, three had mechanical damages (two in arthritic mice, one in CTR). The remaining arthritic tg (n = 6) and WT AIA mice (n = 7), and control tg (n = 9) and WT (n = 8) mice were assessed for histological, histomorphometric and micro-CT analyses.

2.2.2 The K/BxN model

2.2.2.1 K/BxN mouse serum

K/BxN mouse serum was prepared as described by Kouskoff et al. (143). KRN-C57BL/6 mice transgenic for a TCR that recognizes a bovine ribonuclease peptide presented by a MHC class II (as described in the introduction) were crossed with NOD mice to create K/BxN mice which develop arthritis spontaneously. Serum was collected from 60-day-old arthritic K/BxN mice through intracardiac puncture, pooled and stored at -80°C.

As the amount of serum was not sufficient for the experimental set-up and the serum is not commercially available, the group of Prof. Wim van den Berg, University Nijmegen, Netherlands, kindly provided an additional amount of serum. After defrosting sera at room temperature, both batches were mixed and administered in the experimental animals.

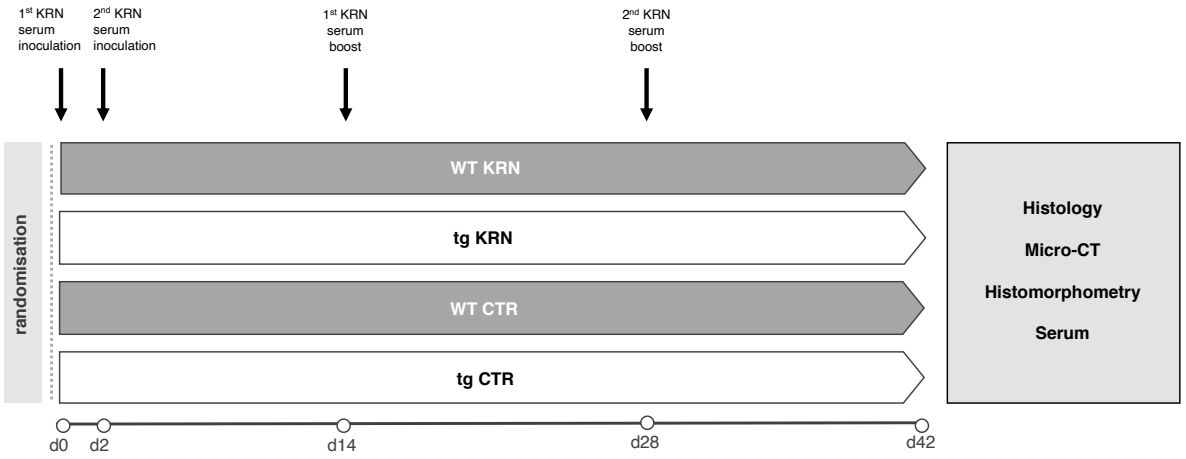

Figure 2: **Experimental design for the long-term K/BxN serum-induced arthritis model**

2.2.2.2 Initiation and prolongation of K/BxN serum-induced arthritis.

Transgenic mice and their wild-type (WT) littermates received intraperitoneal injections of either 150µl of K/BxN mouse serum or phosphate buffered saline (PBS) on days 0 and 2. Injections of control serum were not performed on WT mice, since previous experiments did not show a difference between injection of PBS and control serum (153).

K/BxN serum transfer arthritis was induced in 5-week-old male Col.2.3.-11β-HSD2 transgenic mice (n = 11) and their WT male littermates (n = 11). Four transgenic and nine WT mice served as controls, receiving PBS intraperitoneally. In order to maintain inflammation for a prolonged period, arthritic mice received repeated subcutaneous boost-injections of 150µl K/BxN serum on day 14 and day 28 (Figure 2). In preliminary studies, the dose of 150µl K/BxN serum was compared to 200µl and identified as sufficient in order to maintain a chronically active arthritis (data not shown). Controls received PBS at the same time points. Body weight and clinical parameters of arthritis were assessed daily throughout the entire study. Mice were sacrificed on day 42 after intraperitoneal anesthesia with 75mg/kg body weight ketamine (Cenvet Pty. Ltd., Kings Park, Australia) and 10mg/kg body weight xylazine (Cenvet Pty. Ltd., Kings Park, Australia) by cervical dislocation for histological, micro-CT and histomorphometric analyses.

2.2.2.3 Clinical assessment of arthritis

Daily assessments and measurements were performed at a constant time of day in order to maintain a stable circadian rhythm. Mice were weighed daily. Clinical arthritis was assessed daily by two observers who were blinded to the assigned group and genotype. As previously performed by Buttgereit et al. (153) and described by Lee et al. (157, 158), the severity of arthritis was assessed by applying a clinical score to each paw, with score points assigned as follows: 0 = normal; 1 = mild to moderate swelling and erythematic ankle and/or one swollen digit; 2 = moderate swelling and erythematic ankle or swelling in two or more digits; 3 = marked swelling along all aspects of the paw or all five digits swollen. The sum of scores was calculated with a maximum possible score of 12.

Ankle size measurements were performed daily using a Vernier caliper. The maximum medial-to-lateral diameter was defined at the widest point of each ankle. The average of both ankles (rear paws) was determined and considered for analysis.

2.3 Tissue collection and specimen preparation

Under anesthesia, blood was collected by intracardiac puncture with a 27 gauge needle and a 1ml syringe. After 1 hour, the collected blood was centrifuged for 15 minutes at 8,000 rounds per minute (rpm) and the supernatant stored at -80°C for future serum measurements.

Spleens were carefully prepared from the abdomen and weighed immediately after collection as a potential marker for systemic inflammation (data not shown).

In both AIA experiments (acute AIA and long-term AIA), the right knee joint was dissected for histological analysis. In 5 animals of each arthritic group, knee joints with adjacent synovial tissue and bone marrow were prepared for future RNA analysis and stored at -80°C after immersion in liquid nitrogen. For all AIA mice, regional lymph nodes were extracted from the knee joint and inguinal region. Microarray/PANTHER classifications as well as Proteoanalysis have not yet been performed. The same applies to the collected synovial fluid, which was recovered using a calcium sodium alginate compound (CSAC) method, as described by Seifer et al. (159), where a 2mm circular piece of CSAC, cut using a conventional hole punch, was swabbed into the knee joint cavity, removed, placed in a NUNC tube, and digested with 35µl of sodium

alginate in H_2O (Sigma, Castle Hill, Australia) for 30min at 34°C, followed by the addition of 15μl of 1M Sodium Citrate. The volume was determined by applying the pipette-dialing method (data not shown). Samples were frozen at -80°C and have not been analyzed yet, due to absent significant differences between tg and WT AIA mice in the analysis done so far.
The left tibia was prepared for micro-CT and histomorphometry.

For the K/BxN serum-transfer model, the right hind paws and tibia of each mouse were dissected for histological and histomorphometric analysis, while the left hind paw and tibia were harvested for micro-CT analysis.
All samples for histological and micro-CT analysis were fixed and stored in 4% paraformaldehyde/PBS.

2.4 Histology

2.4.1 Sample preparation

Samples were fixed in paraformaldehyde 4% (PFA extra pure, Merck KGaA, Darmstadt, Germany) and buffered with 0.1 moL/L phosphate buffer (AMRESCO Inc., Solon, OH, USA; pH 7.4) for 48 hours at 4°C. For histological analysis, samples were decalcified with 10% ethylenediaminetetraacetic acid (EDTA, Fronine Laboratory Supplies, Taren Point, Australia, pH 7.0) for 21 days at 4°C on a rocking shaker. EDTA was changed twice weekly.
Following decalcification, tissues were embedded in paraffin with an Automatic Tissue Processor (Leica TP 1020, Leica Microsystems GmbH, Wetzlar, Germany) by dehydration in rising ethanol concentration (50 v/v% for 4 hours, 70 v/v% for 4 hours, 95 v/v% for 4 hours, 100% for 2 hours, 100% for another 2 hours, 100% vacuum for 2 hours) and xylene clearing (vacuum for 2 hours, no vacuum for 2 hours, another 2 hours vacuum). Embedding was performed with double filtered paraffin wax (Paraplast Tissue Embedding Medium, Tyco Healthcare Group, Mansfield, MA, USA).

For the AIA experiments, the knee joint was positioned with slight flexion and sagittal sections were collected at the menisci level.

For the K/BxN long-term experiment, ankle joints were analyzed in the transverse axis, centering the lateral ankle joint at a mid-talar view.

Serial 4-*µ*m sections were obtained using a Leica microtome (Leica Microsystems GmbH, Wetzlar, Germany). Sections were mounted onto microscope slides coated with 3-aminopropyltriethoxysilane (AES, Sigma-Aldrich Inc., St. Louis, MO, USA) and consecutively stained with hematoxylin and eosin (H&E) for general histologic evaluation, with toluidine blue for assessment of cartilage degradation and proteoglycan loss. To identify osteoclasts and bone erosions, sections were stained for tartrate-resistant acid phosphatase (TRAP).

2.4.2 Slide Coating

Microscope slides were immersed in 60°C warm water and detergent seven times for two hours. After rinsing with filtered cold tap water for five minutes, the slides were again washed with distilled water twice for five minutes. After that, the microscope slides were soaked in 80 v/v% ethanol for two hours and dried overnight. Subsequently, the slides were dipped briefly in AES solution (2%) for 30 seconds, and afterwards briefly in acetone and distilled water, and then dried at room temperature overnight.

The paraffin was melted on a hot plate at 60°C for 10 minutes, dewaxed with three changes of xylene immersion for five minutes each, and rehydrated with three changes of 80% ethanol for three minutes each. The slides were rinsed with tap water before staining.

2.4.3 Histologic staining

2.4.3.1 Hematoxylin and Eosin (H&E)

H&E staining was performed for general histologic analysis of inflammation. Hematoxylin (Lillie-Mayer's hemalum solution, Fronine Laboratory Supplies, Taren Point, Australia) and eosin (Eosin Y, Fronine Laboratory Supplies, Taren Point, Australia) were used according to the following protocol. Slides were stained in hematoxylin for 7 minutes, thoroughly rinsed with tap water, then stained with eosin for ten minutes, followed by three changes of 100% ethanol for three minutes each, and

two changes of xylene (two minutes each). The stained slides were mounted with a coverslip with DEPEX (BDH Ltd., Poole, UK).

2.4.3.2 Toluidine blue

In order to assess the degree of cartilage degradation and proteoglycan loss, staining with toluidine blue was performed. Slides were immersed into toluidine blue solution (0.1%) for eight minutes, subsequently rinsed with tap water, dried overnight at 37°C and mounted with a DEPEX coverslip (see above).

2.4.3.3 Tartrate-resistant acid phosphatase (TRAP)

For the assessment of focal bone resorption at the arthritic site, TRAP staining was applied for identifying osteoclasts and bone erosions. TRAP buffer consisting of sodium acetate anhydrous (50mM, Fluka Chemie AG, Basel Switzerland), along with potassium sodium tartrate (40mM, Sigma-Aldrich Inc., St. Louis, MO, USA) was mixed with Milli-Q water and titrated to an acidic pH of 5.0 with glacial acetic acid. Naphthol AS-MX (Sigma-Aldrich Inc., St. Louis, MO, USA) was added as a substrate and Fast Red Violet Luria Bertani salt (Sigma-Aldrich Inc., St. Louis, MO, USA) as a detection agent for the reaction product. Prepared mounted microscope slides were covered in TRAP stain in darkness for 0.5 – 1 hours, depending on the degree and rate of the reaction as expressed by color intensity. Slides were then rinsed with tap water to halt the reaction and counterstained with a 1:10 v/v solution of Harris No. 3 hematoxylin (Fronine Laboratory Supplies, Taren Point, Australia) in Milli-Q water for 30 seconds. Finally, slides were rinsed with tap water and dried overnight.

2.4.4 Histopathologic Scoring

2.4.4.1 Inflammation

For the AIA models, H&E staining was used to assess synovial inflammation by two independent and blinded observers. Applying an established semi-quantitative scoring system (160, 161), inflammation was graded for aspects of synovitis, joint space exudate and soft tissue inflammation on a 0-3 scale, with 0 representing no change and 1 through 3 indicating mild to severe changes.

For the K/BxN model, histological assessment of H&E stained samples was performed by two independent and blinded observers for inflammatory cell infiltration, synovial lining cell hyperplasia and soft tissue edema according to an established semi-quantitative scoring system (153, 157). Inflammatory activity was scored on a 0-5 point scale as follows: 0 = normal; 1 = minimal inflammatory infiltration; 2 = mild infiltration with no soft tissue edema or synovial lining cell hyperplasia; 3 = moderate infiltration with surrounding soft tissue edema and some synovial lining cell hyperplasia; 4 = marked infiltration, edema, and synovial lining cell hyperplasia; 5 = severe infiltration with extended soft tissue edema and marked synovial lining cell hyperplasia.

2.4.4.2 Cartilage degradation

For the AIA models, cartilage degradation was quantified using the above-mentioned score on a 0-3 scale.

Similarly, for K/BxN, cartilage damage was scored on a 0-5 point scale as follows: 0 = normal; 1 = loss of toluidine blue staining only; 2 = loss toluidine blue staining and moderate multifocal cartilage loss; 3 = moderate loss of toluidine blue staining and moderate multifocal cartilage loss; 4 = marked loss of toluidine blue staining with marked multifocal cartilage loss; 5 = severe multifocal cartilage loss.

2.4.4.3 Bone erosions

For the AIA experiments, a scale of 0-3 was used as mentioned above to assess bone erosion severity.

For the K/BxN experiment, assessment of bone erosions was according to a 0-5 point scale: 0 = no erosions; 1 = minimal (not readily apparent on low magnification, rare osteoclasts visible); 2 = mild (not readily apparent on low magnification, some osteoclasts visible); 3 = moderate (obvious bone resorption with numerous osteoclasts visible); 4 = marked (large erosion areas extending into the bone cortex with numerous osteoclasts visible in all areas); 5 = severe erosions with full thickness defects in the cortical bone.

2.5 Micro-CT

2.5.1 Micro-CT scanning

In order to assess local structural damage of bone as well as systemic bone loss induced by the inflammatory process, a micro-CT based analysis of the ankle joints (in the K/BxN experiment) and tibia was performed, respectively. A Skyscan 1172 X-ray microscope (Bruker microCT, Kontich, Belgium) was used for microtomography, scanning the tibiotalar joint and tibia. Specimen were placed in a specimen holder and a 1mm aluminium foil was attached as a correction filter to minimize the effect of beam hardening and artefacts in the processed images. Scanning was done at 100kV and 100μA, and at an exposure time of 590 ms. For the tibia, 1,800 projections were created at a resolution of 6.93 μm/pixel. For the reconstruction of sections, a modified Feldkamp cone-beam algorithm was used, with beam hardening correction set to 50%. In order to create 3D visualization from the reconstructed right ankles in the K/BxN serum-transfer model, VGSTUDIO MAX 1.2 software (Volume Graphics GmbH, Heidelberg, Germany) was used. All analyses were performed using CTAnalyser software version 1.8 (Skyscan, Bruker microCT, Kontich, Belgium).

2.5.2 Micro-CT analysis

To quantify systemic bone loss, trabecular morphometry of the proximal tibia was assessed, selecting a 1mm (= 132 slides) long region of interest (ROI) exactly 0.5 mm (66 slides) below the growth plate and within the endosteal borders. The greyscale was set from 75 to 255 percent. The selected ROI served for determining the volume of interest and thus for the quantification of the following morphometric parameters: bone volume fraction (BV/TV = bone volume / tissue volume, in %); trabecular number (Tb.N., /mm), trabecular thickness (Tb.Th., μm), trabecular separation (Tb.Sp., μm).

For assessment of the degree of local bone erosions at the direct arthritic sites of the ankles, three-dimensional as well as two-dimensional micro-CT images were used applying a semi-quantitative score, defining a region of interest encompassing tibia, fibula, calcaneus and talus. For the two-dimensional images, a region 1 mm proximal to the beginning of the tibiotalar joint was selected. Assessment was performed as follows by two independent observers: 0 = no erosions or cortical damage; 1 = mild

cortical bone transformation and erosive changes beginning; 2 = moderate bone damage comprising erosions and vacuoles; 3 = severe damage with large erosions and full-thickness defects of the corticalis.

A total score of the average of the two observers was used, comprising the assessment of both two- and three-dimensional images.

2.6 Histomorphometric analysis

Bone histomorphometry was performed on the left proximal tibial metaphysis for analysis of systemic bone changes. H&E staining was used to identify osteoblasts and osteocytes as indicators of bone formation, while TRAP staining was used to identify osteoclasts as a measure of bone resorption. Osteomeasure XP v. 3.2.1.5 (Osteometrics, Inc., Decatur, GA, USA) was used in conjunction with a light microscope (Leica Microsystems GmbH, Wetzlar, Germany). A region of interest was selected 0.3 mm below the growth plate of the tibia comprising an area of 1.5 x 1 mm. The following measurements were performed at a 400-fold magnification:

BV/TV: bone volume/tissue volume (%), indicating the percentage of bone at the trabecular region of interest.

BS/BV: bone surface/bone volume (%), indicating the percentage of bone surface at the recently formed bone.

Ob.S/BS: osteoblast surface/bone surface (%), indicating the percentage of bone surface covered with osteoblasts.

N.Oc/BS: number of osteoclasts/bone surface (per mm), indicating the mean number of osteoclasts per mm bone surface.

Oc.S/BS: osteoclast surface/bone surface (%), indicating the percentage of bone surface covered with osteoclasts.

2.7 Measurements of serum cytokines

For the AIA model only, serum levels of inflammatory cytokines were analyzed with the cytometric bead array technique at days 14 and 28. Pre-mixed cytokine assays (Bio-Rad, Munich, Germany) were used according to the manufacturer, and data was collected by applying the Bio-Plex suspension system (Bio-Rad, Munich, Germany). Each sample was read in duplicate and measured against the mean of two dilution

rows. When values were found to be below the detection limit, a value half of the detection limit was assigned for analysis. The following inflammatory cytokines were analyzed: TNF-α, IFN-γ, murine IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10,IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-18, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), 1β (MIP-1β) and 2 (MIP-2), regulated upon activation normal T cell expressed and secreted (RANTES), keratinocyte-derived cytokine (KC), monokine induced by interferon-gamma (MIG), Eotaxin, leukemia inhibitory factor (LIF), basic fibroblast growth factor (basic-FGF), platelet-derived growth factor homodimer (PDGF-BB), vascular endothelial growth factor (VEGF), granulocyte colony-stimulating growth factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF).

2.8 Statistical analysis

For comparison of normally distributed data between two groups, the *t*-test was used. For non-normally distributed data, comparison of two experimental groups was done using the Mann-Whitney test. When multiple comparisons for the same parameter were planned, multiple two-group comparisons were conducted only if the Kruskal-Wallis test was significant over all 4 groups, and in these cases, the Bonferroni correction was used to adjust the level of significance to a* = 0.0125 for four parallel tests (WT-CTR vs tg-CTR, WT-arthritic vs tg-arthritic, WT-CTR vs WT-arthritic, tg-CTR vs tg-arthritic). Values are stated as mean values and standard deviation, unless stated otherwise.

Clinical data for body weight, clinical score, and joint diameter over the entire experimental course was modelled by a generalized linear model with repeated-measure analysis. In order to control for the growth processes, especially in the long-term models, repeated-measures analyses were corrected for growth rate and tested for confounders such as ankle size and body weight on day 0.

The growth rate was calculated from the linear correlation of a time dependent growth of ankle size over body weight gain of the control animals. The calculated growth effect was then subtracted from the ankle size (arthritis effect = overall effect as measured by the ankle size / knee diameter minus growth effect). The Mauchly test was used to

determine sphericity. If the latter was significant, sphericity was abandoned and the Greenhouse-Geisser p-value was used. In case of non-significance of the Mauchly test, sphericity was assumed and the respective p-value was used.

With the AIA model, body weight at day 0 and body weight gain during the experimental course was similar between tg and WT arthritic mice. With the KRN long-term model, body weight as well as ankle size on day 0 were significantly lower in tg KRN compared to WT KRN, so that repeated-measures analysis was corrected for growth rate and ankle size growth, using day 0 as the adjusting covariant. P_{KRN} and P_{AIA} represent differences in the time course of transgenic and WT arthritic mice as calculated from the repeated-measures analysis by interaction day x group of within-subject effects.

IBM SPSS Statistics version 19 (IB, Armonk, NY, USA) was used for statistical analysis, with p-values less than 0.05 considered significant.

3 RESULTS

3.1 Acute AIA

3.1.1 Body weight

At baseline, after immunization, the body weights of eight-week-old tg mice and their WT littermates were similar (mean ± SD: 36.9 ± 3.1 g vs 37.6 ± 3.6 g; P = 0.454). Over the 14-day course of arthritis, the gain in body weight was also similar in arthritic vs control mice [P = 0.077] as well as for tg AIA compared to WT AIA mice [P_{AIA} = 0.908].

3.1.2 Knee joint swelling

Prior to induction of arthritis, knee joint diameter did not differ between tg and WT mice (mean ± SD: 4.057 ± 0.209 mm vs 4.058 ±0.206; P = 0.979]. After intra-articular injection of mBSA, acute arthritis developed in both tg and WT mice, with significant knee joint swelling compared to control mice [P < 0.001]. Peak values were reached on day 1 post injection, with a mean increase of joint diameter of 0.96 ± 0.58 mm for tg mice and 1.07 ± 0.53 mm for WT mice. After day 1, knee joint swelling gradually resolved over the course of 14 days in both groups. No statistically significant difference in knee joint swelling was observed between tg and WT mice [P_{AIA} = 0.468] (Figure 3).

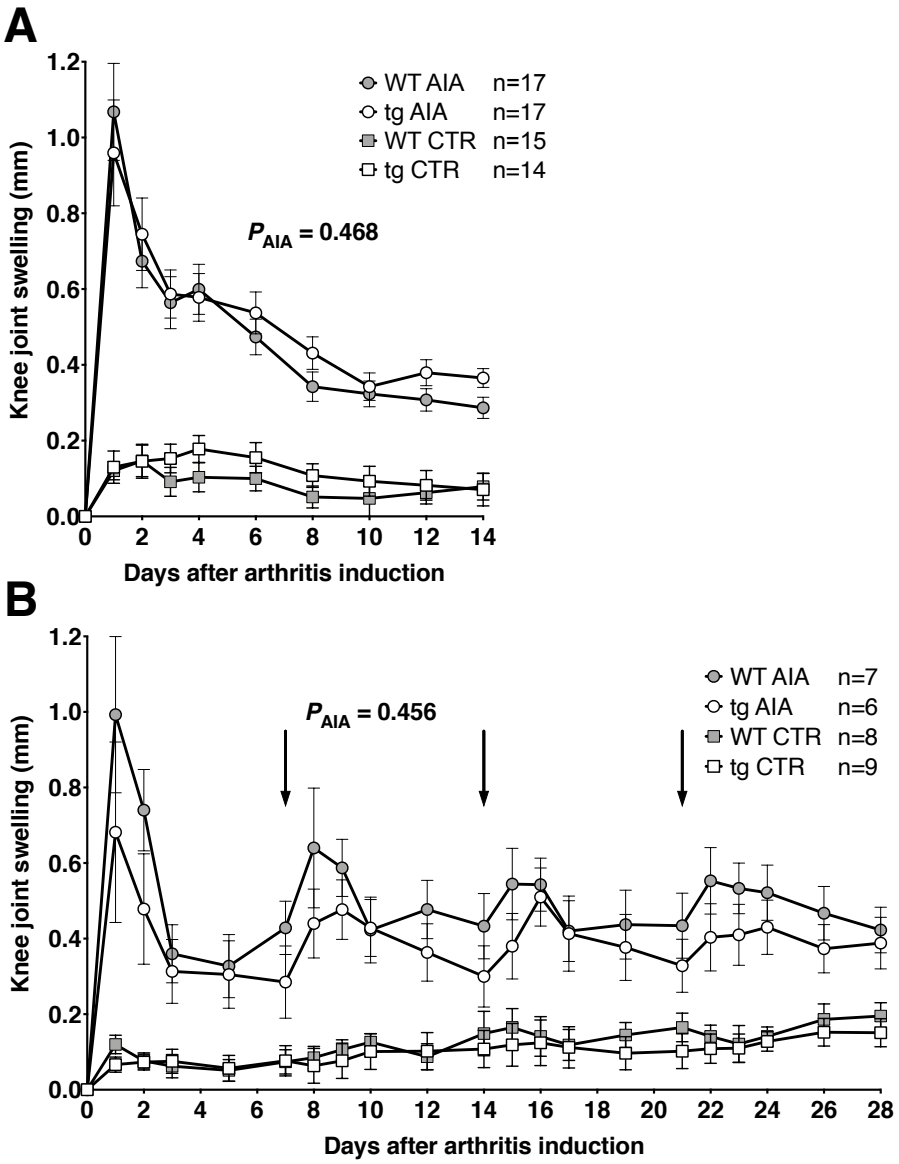

Figure 3: **Clinical assessment of inflammation in acute and long-term AIA**
Arthritic mice received mBSA injections on day 0 (AIA), control mice (CTR) received phosphate buffered saline (PBS). **(A)** Means and SEM for knee joint swelling from day 1 to day 14 post injection. P_{AIA} value indicates the significance of the difference in variation of knee joint swelling over time between transgenic (tg) and wild-type (WT) mice receiving mBSA. **(B)** Means and SEM for knee joint swelling from day 1 to day 28, after intra-articular injection of mBSA on day 0 and subsequent flare-up reactions from intravenous mBSA injections on days 7, 14, 21 (arrows). The P_{AIA} value represents the significance after repeated-measures analysis between tg and WT AIA mice.

3.1.3 Histopathological assessment of arthritis

The findings of clinical arthritis were also reflected in histopathological analyses of inflammation, cartilage degradation and bone erosion. At day 14 post injection, indicators of inflammatory activity were significantly higher in AIA mice compared to control mice, with overall moderate to severe inflammation, as expressed by the total histology score (Figure 4). However, inflammatory activity did not differ between tg AIA mice compared to WT AIA mice, and also when looking at single parameters of

inflammation such as synovitis, joint space exudate and soft tissue inflammation (Figure 4). Also, no statistically significant difference was observed in terms of cartilage degradation and proteoglycan loss between both arthritic groups (Figure 4). For bone erosions as a hallmark of profound arthritis, which were overall mild to moderate, again no difference between tg AIA and WT AIA was found (Figure 4).

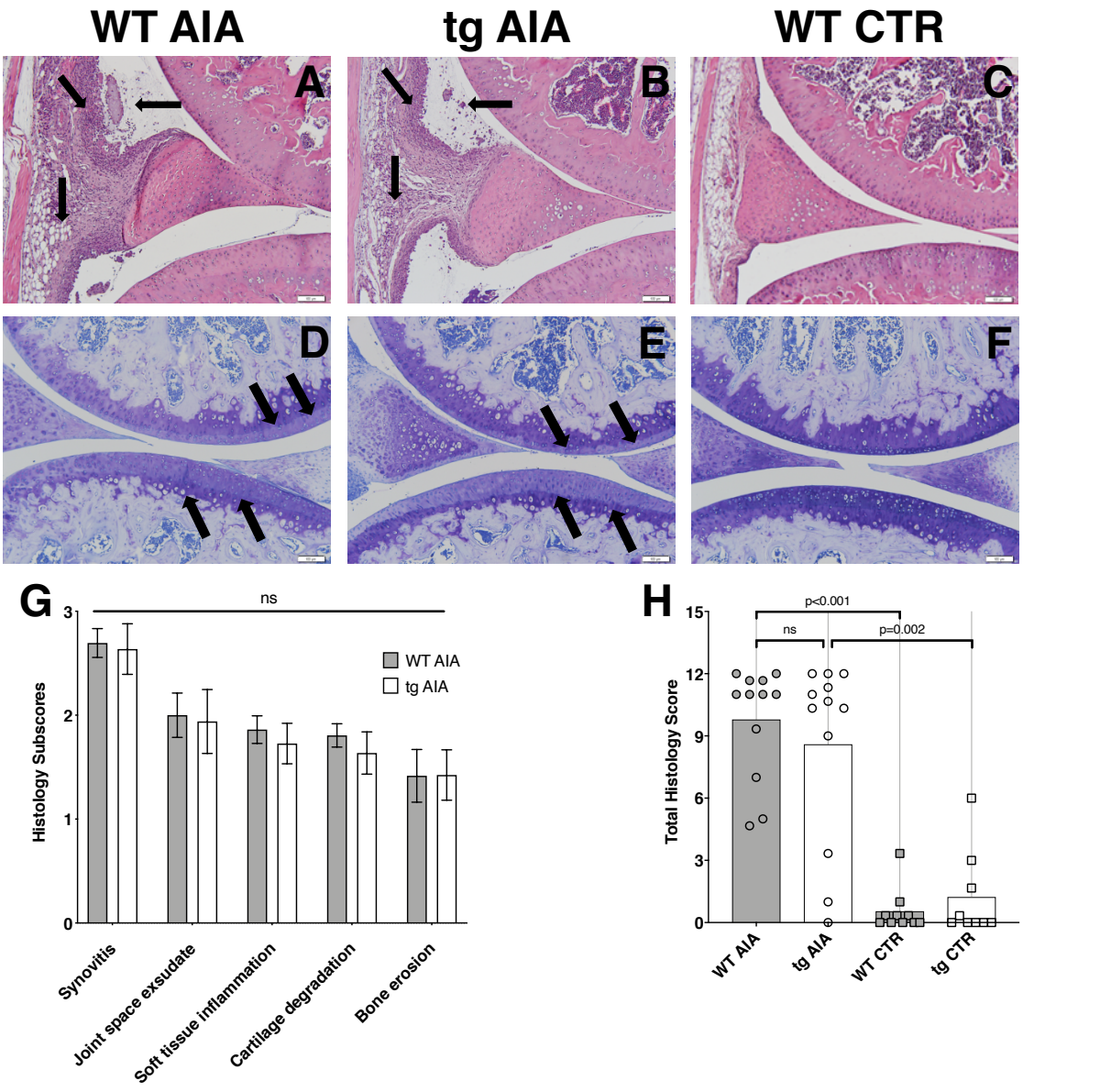

Figure 4: **Arthritis and cartilage damage in the knee joint 14 days post intra-articular injection**
(A-F) Representative histologic sections of knee joints from transgenic (tg) and wild-type (WT) mice after intra-articular injection with mBSA (AIA) and non-arthritic control mice (CTR). Inflammatory activity and cartilage damage were similar in tg and WT arthritic mice. **(A-C)** Hematoxylin and eosin staining. **Arrows** indicate synovitis, joint space exudate, and soft tissue inflammation. Bars = 100 μm. **(D-F)** Toluidine Blue staining. **Arrows** indicate proteoglycan loss of articular cartilage. Bars = 100 μm. **(G-H)** Histopathologic score (G subscores, H total) in AIA mice, 14 days post mBSA injection. Bars show mean ± SEM or scatter dot plot, respectively. Findings in arthritic tg and WT AIA mice for subscores were analyzed by Mann-Whitney test. Total scores were compared between the four groups with adjustment for 4 parallel tests ($\alpha^* = 0.0125$). ns = not significant.

3.1.4 Micro-CT

Although the AIA model represents an arthritis model in which only one single joint is affected, potential systemic effects of inflammation on bone due to the involvement of the adaptive immune system were analyzed and quantified by micro-CT of the contralateral (=left) tibia – a region distant from the actual site of inflammation. There was no statistically significant difference between either arthritic and control mice, or between tg and WT mice, in terms of bone volume fraction expressed by (BV/TV), trabecular number and separation, implying that AIA, by being restricted to the knee joint, does not affect bone morphology systematically after 14 days. Trabecular thickness (Tb.Th.) was significantly higher in tg control mice compared to their WT littermates, although this finding remains somewhat inconclusive and unspecific with regard to the other parameters of bone morphology (Figure 5).

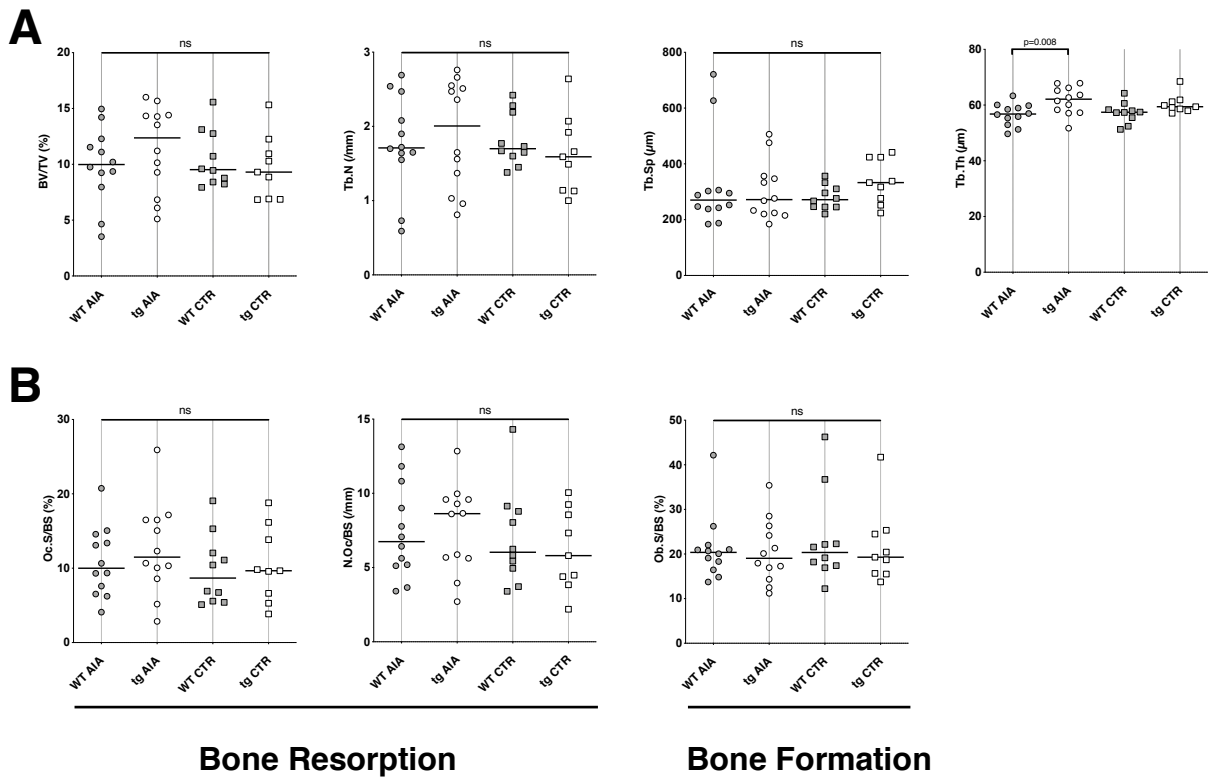

Figure 5: **Micro-CT and histomorphometric analysis of the contralateral tibia 14 days post injections of mBSA**

Bone turnover was measured at a site remote from active arthritis to assess systemic effects of inflammation on bone. **(A)** Micro-CT. The bone volume fraction (bone volume/tissue volume [BV/TV]), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th) are shown for bones harvested. Bars show the mean ± SEM. Findings between the 4 groups were compared using Mann-Whitney test with adjustment for 4 parallel tests (α^*=0.0125). ns = not significant **(B)** Histomorphometric quantification. Bone resorption, shown as osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone surface (NOc/BS), respectively. Bone formation, shown as osteoblast surface/bone surface (Ob.S/BS). Scatter dot plot with mean. Findings between the 4 groups were compared by Mann-Whitney test with adjustment for 4 parallel tests (α^*=0.0125). ns = not significant.

3.1.5 Bone histomorphometry

Histomorphometric analysis of the contralateral (left) tibia corroborated the findings by micro-CT, which showed no systemic effects of AIA on bone morphology. In terms of bone resorption, mean osteoclast number and osteoclast surface per bone surface did not differ between arthritic and control mice, or between tg and WT mice (Figure 5). The same was observed for markers of bone formation as indicated by osteoblast surface per bone surface (Ob.S/BS), where no difference was found between tg and WT, or between AIA and control mice. Thus, systemic bone turnover does not seem to be affected by monoarticular inflammation.

3.1.6 Serum levels of cytokines

With the AIA model involving the adaptive immune system, serum levels of cytokines as specified above were measured (a total of 32). While there was a statistically significant difference between tg AIA and WT AIA mice for IL-1α, IL-12 (p70), and IL-13 on day 14, no statistical difference between AIA and control mice could be observed. For the remaining cytokines (TNF-α, IL-1β, IL-9, IL-10 (p40), IL-15, IL-18, MCP-1α, MIP-1β, RANTES, KC, MIG, Eotaxin, LIF, basic-FGF, PDGF-BB, VEGF, M-CSF or G-CSF) there were also no differences detected between arthritic vs control and tg vs WT mice, respectively. Serum concentrations of IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-17A, MIP-2, and GM-CSF were mostly below the detection limit (Table 1).

Table 1: **Serum cytokine levels in control mice and arthritic AIA mice**

	control mice, day 14				AIA mice, day 14			
	wild-type (n=15)		transgenic (n=14)		wild-type (n=17)		transgenic (n=17)	
TNF-α (pg/ml)	36	(36-408)	36	(36-36)	409	(36-476)	36	(36-341)
IL-1α (pg/ml)	67	(58-71)	5	(5-64)	89	(54-97)	44	(5-66) ♯
IL-1β (pg/ml)	558	(46-1050)	46	(46-1097)	574	(46-1397)	46	(46-431)
IL-6 (pg/ml)	6	(6-6)	6	(6-6)	6	(6-6)	6	(6-6)
IL-10 (pg/ml)	50	(5-111)	5	(5-61)	72	(46-107)	50	(5-64)
IL-12 (p40) (pg/ml)	175	(114-222)	131	(114-183)	193	(147-272)	152	(130-224)
IL-12 (p70) (pg/ml)	149	(13-202)	108	(13-149)	162	(123-236)	108	(13-130) ♯
IL-13 (pg/ml)	45	(45-964)	45	(45-523)	852	(361-1470)	45	(45-45) ♯
M-CSF (pg/ml)	204	(119-593)	548	(158-612)	295	(115-481)	488	(146-608)
G-CSF (pg/ml)	121	(13-257)	59	(13-140)	165	(13-216)	105	(13-189)
	control mice, day 28				AIA mice, day 28			
	wild-type (n=8)		transgenic (n=9)		wild-type (n=7)		transgenic (n=6)	
TNF-α (pg/ml)	1361	(903-2310)	1255	(655-1468)	1255	(655-1468)	1577	(848-1685)
IL-1α (pg/ml)	9	(9-9)	9	(9-9)	9	(9-9)	9	(9-9)
IL-1β (pg/ml)	14	(14-144)	14	(14-14)	14	(14-979)	517	(14-1073)
IL-6 (pg/ml)	6	(6-6)	6	(6-6)	6	(6-6)	6	(6-6)
IL-10 (pg/ml)	56	(49-68)	44	(44-54)	49	(40-54)	68	(35-130)
IL-12 (p40) (pg/ml)	98	(90-136)	70	(56-103)	110	(81-147)	83	(72-90)
IL-12 (p70) (pg/ml)	8	(8-8)	8	(8-8)	8	(8-8)	86	(8-105) §, ♯
IL-13 (pg/ml)	101	(8-253)	8	(8-8)	160	(113-254)	254	(66-1374) §
M-CSF (pg/ml)	95	(85-103)	106	(81-112)	86	(79-120)	88	(77-470)
G-CSF (pg/ml)	12	(12-12)	12	(12-12)	12	(12-12)	12	(12-12)

Values indicated are the median values (interquartile range). Findings between groups were compared using the Kruskal-Wallis test, followed by multiple two-group comparisons. The Bonferroni correction was used to adjust the significance level for 4 parallel comparisons (α* = 0.0125). TNF-α = tumor necrosis factor α; IL-1α = interleukin-1α; M-CSF = macrophage colony-stimulating factor; G-CSF = granulocyte colony-stimulating factor.
Wild-type versus transgenic CTR: none
wild-type versus transgenic AIA mice: IL-1α $p_{14} = 0.008$; IL-12 (p70) $p_{14} = 0.010$ and $p_{28} = 0.009$; IL-13 $p_{14} = 0.001$
CTR versus AIA wild-type: none
§ CTR versus AIA transgenic: IL-12 (p70) $p_{28} = 0.001$; IL-13 $p_{28} = 0.008$

3.2 Prolonged arthritis model

In acute AIA, the course of arthritis naturally and gradually resolves after reaching a peak at day 1. Bearing in mind that in humans rheumatoid arthritis is a chronically active disease with frequent flares, we tried to simulate a prolonged course of monoarthritis resembling the natural course of RA by repeating antigen exposure weekly through intravenous mBSA injections, thus maintaining inflammation for a period of 28 days.

3.2.1 Body weight

Unlike in acute AIA, body weights at baseline were significantly lower in tg mice compared to WT mice (mean ± SD: 36.1 ± 1.4 g vs 38.6 ± 3.4 g; P = 0.016). With each flare-up reaction, a minor weight loss of <10% was observed in both arthritic and control mice [P = 0.314], implying the effect as being possibly due to a stress reaction. Comparing arthritic tg to WT mice, the degree of weight loss was similar [P = 0.553].

3.2.2 Knee joint swelling

At baseline, knee joint swelling did not differ between tg and WT mice (mean ± SD: 3.85 ± 0.08 mm versus 3.88 ± 0.07 mm; P = 0.203). Upon induction of arthritis by intra-articular injection of mBSA, significant arthritis developed in both tg and WT mice [P < 0.001], as was observed in acute AIA with a maximum peak at day 1 followed by a gradual subsiding of swelling until flare-up reactions were induced on days 7, 14 and 21, resulting in corresponding peaks of knee joint swelling the following day. Despite the flare reactions, no statistically significant difference in knee joint swelling was observed between tg and WT AIA mice. [P_{AIA} = 0.456], (Figure 3). Clinically, the effect of boost injections seemed to become less pronounced with each flare, hinting at mechanisms of desensitization for the systemic induction of flare injections.

3.2.3 Histopathological assessment of inflamed joints

Similar to acute AIA, no differences were observed with regard to histologic indices of inflammatory activity, cartilage degradation and bone erosions at day 28 between tg and WT arthritic mice (Figure 6). Overall, maximum scores in the prolonged AIA model reached a comparatively lower degree for all individual indices of histologic

assessment compared to the acute AIA model, and especially concerning bone erosions. As the prolonged AIA model was conducted in an independent set of experiments, no direct comparisons and statistical assumptions can be made between the prolonged AIA and acute AIA models. Yet it would seem that, in line with the subsiding effect of repeated boost injections on knee joint swelling, inflammatory processes were maintained at a mild level only, inducing rather mild flares.

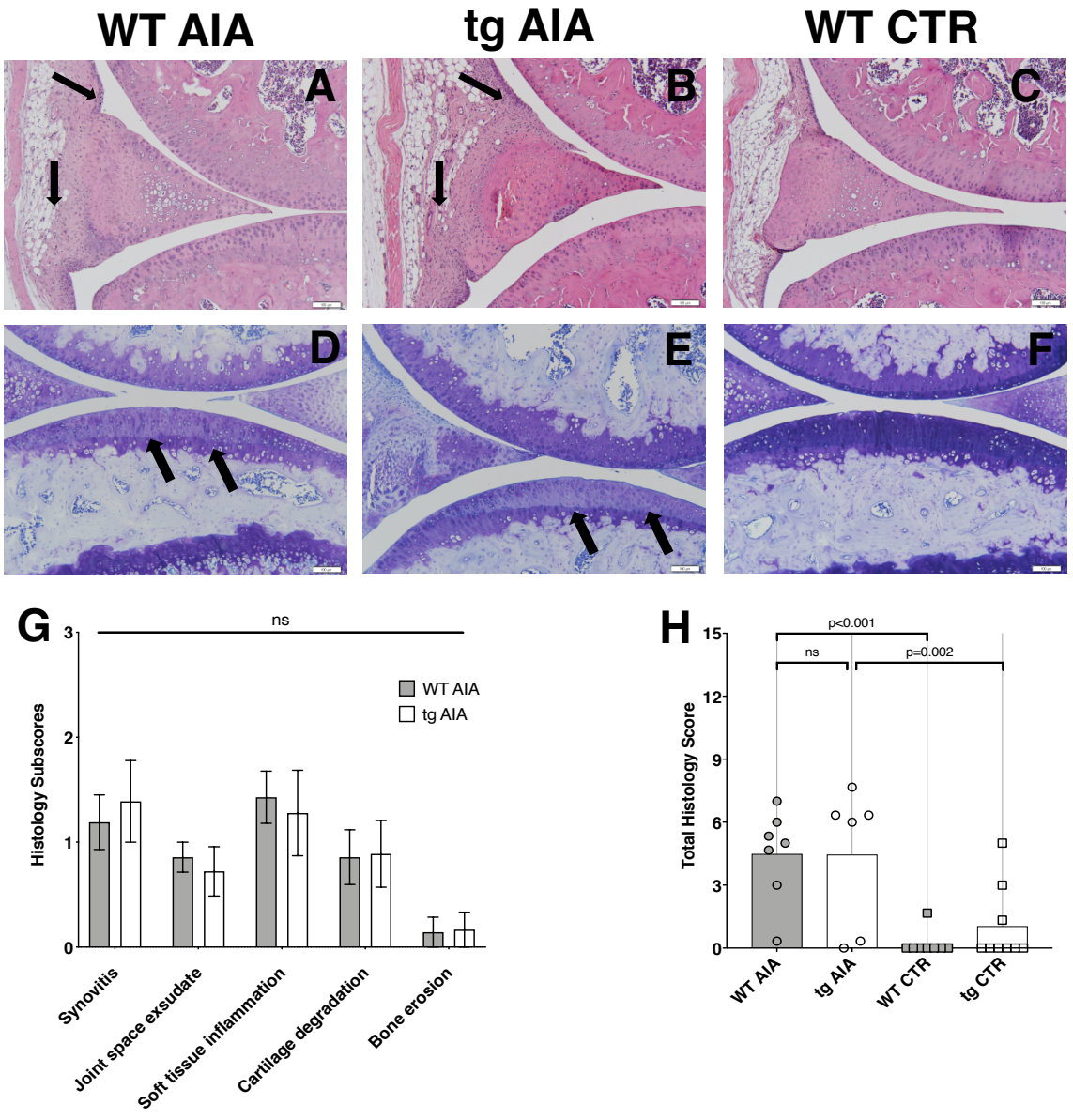

Figure 6: **Arthritis and cartilage damage in the knee joint on day 28 of long-term AIA**
Mice received intra-articular injection on day 0 and three intravenous injections with mBSA on days 7, 14, and 21 to induce flares. **(A-F)** Representative histologic sections of knee joints from tg and WT arthritic mice (AIA) and controls (CTR). Inflammation and cartilage degradation did not differ between tg and WT AIA mice. **(A-C)** H&E staining. **Arrows** indicate synovitis and soft tissue inflammation. Bars = 100μm. **(D-F)** Toluidine blue staining. Arrows indicate proteoglycan loss of articular cartilage. Bars = 100 μm. **(G-H)** Histopathological assessment (G subscores, H total) in AIA mice 28 days post initial intra-articular injection and three flare-up injections. Bars show the mean ± SEM or scatter dot plot, respectively. Findings in tg and WT AIA for subscores were compared by Mann-

Whitney test. Total scores were compared between the 4 groups with adjustment for 4 parallel tests ($\alpha^* = 0.0125$). ns = not significant.

3.2.4 Micro-CT

In line with acute AIA, no statistically significant differences between arthritic and control mice were found, regardless of genotype (Figure 7), except for trabecular thickness, which was significantly greater in tg AIA mice compared to WT AIA mice, indicating that systemic effects on bone morphology also did not occur in the prolonged model.

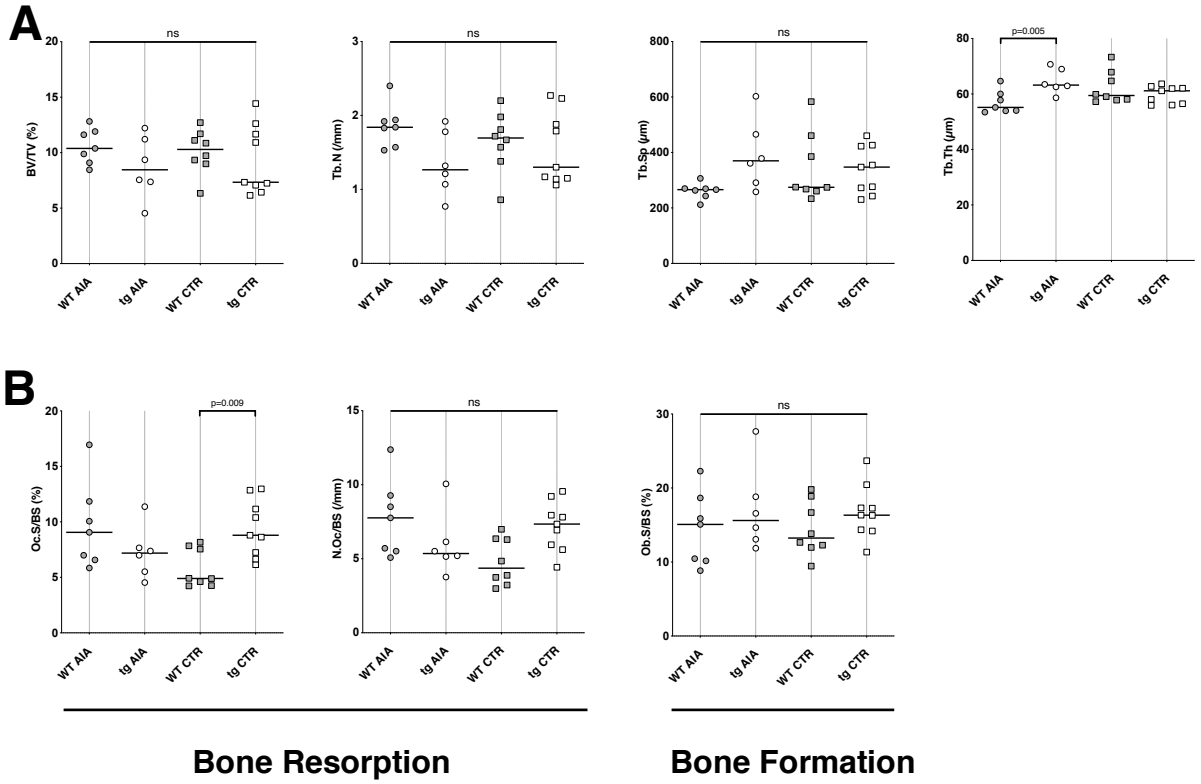

Figure 7: **Micro-CT and histomorphometric analysis of the contralateral tibia in the long-term AIA experiment** Bone turnover was measured at a site remote from active arthritis to assess systemic effects of inflammation on bone. **(A)** Micro-CT. The bone volume fraction (bone volume/tissue volume [BV/TV]), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th) are shown for bones harvested. Bars show the mean ± SEM. Findings between the 4 groups were compared using Mann-Whitney test with adjustment for 4 parallel tests ($\alpha^*=0.0125$). ns = not significant. **(B)** Histomorphometric quantification. Bone resorption, shown as osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone surface (NOc/BS), respectively. Bone formation, shown as osteoblast surface/bone surface (Ob.S/BS). Bars show the mean ± SEM. Findings between the 4 groups were compared by Mann-Whitney test with adjustment for 4 parallel tests ($\alpha^*=0.0125$). ns = not significant

3.2.5 Bone histomorphometry

Concerning bone turnover, as measured by histomorphometry, no statistically significant differences were observed between arthritic and control mice. Within the

control group, tg mice had significantly higher mean osteoclast surface per bone surface without higher mean osteoclast numbers. Tg AIA mice did not differ in that regard from WT AIA mice in the prolonged AIA model.

3.2.6 Serum levels of cytokines

By systemically inducing a local flare-up reaction in contrast to the acute AIA model, a systemic response was assumed to be reflected by cytokine levels in the serum. Although there was a statistically significant difference between tg AIA and WT AIA mice for IL-12 (p70) on day 28 (Table 1), a significant arthritis effect was only observed for IL-12 and IL-13 between tg AIA compared to tg control mice, while the corresponding WT mice did not show a difference in cytokine levels. No differences were observed for the other cytokines when above the detection limit. The following cytokines were below the expression limit: IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-17A, MIP-2, GM-CSF, MCP-1, LIF and G-CSF.

3.3 K/BxN Long-term model

3.3.1 Body weight

Prior to induction of arthritis, body weight was significantly lower in tg mice (mean ± SD: 24.6 ± 2.3 g) compared to WT mice (mean ± SD: 28.0 ± 2.5 g; $p < 0.001$). Following induction of arthritis, weight gain was similar in transgenic and WT mice, regardless of arthritis phenotype.

3.3.2 Ankle size

Related to the lower body weight of tg mice at day 0, differences were also seen in ankle size prior to induction of arthritis, with significantly lower ankle sizes in tg than WT mice (mean ± SD: 3.08 ± 0.08 g and 3.14 ± 0.09 g, respectively; $p = 0.03$), but without a significant independent effect on the overall course of ankle size. Correcting for the growth rate, no significant difference was detected in ankle size between tg and WT mice over the entire course of 42 days with repeated boost injections [$P_{KRN} = 0.697$] (Figure 8A). In contrast to the acute AIA model, arthritis increased gradually and peaked approximately around day 8, as seen in previous studies. Boost injections maintained arthritis at a stable level, however, similarly to the prolonged AIA model, it appeared that the effect of an additional boost at day 28 seemed to be somewhat ameliorated in terms of maintaining only a mild degree of inflammation.

3.3.3 Clinical arthritis score

Since in K/BxN serum-induced arthritis (unlike in AIA), multiple joints are affected by arthritis, a clinical score was applied to also consider inflammatory activity of joints not measured by a caliper. There was no statistically significant difference between tg and WT KRN mice [$P_{KRN} = 0.150$] for the clinical score involving all four paws (Figure 8B). Since a level of significance could not be reached, a statistical analysis of single time points was not permitted, although it seems that after a boost injection on day 14, arthritis continued to be less pronounced in tg KRN mice compared to their WT littermates.

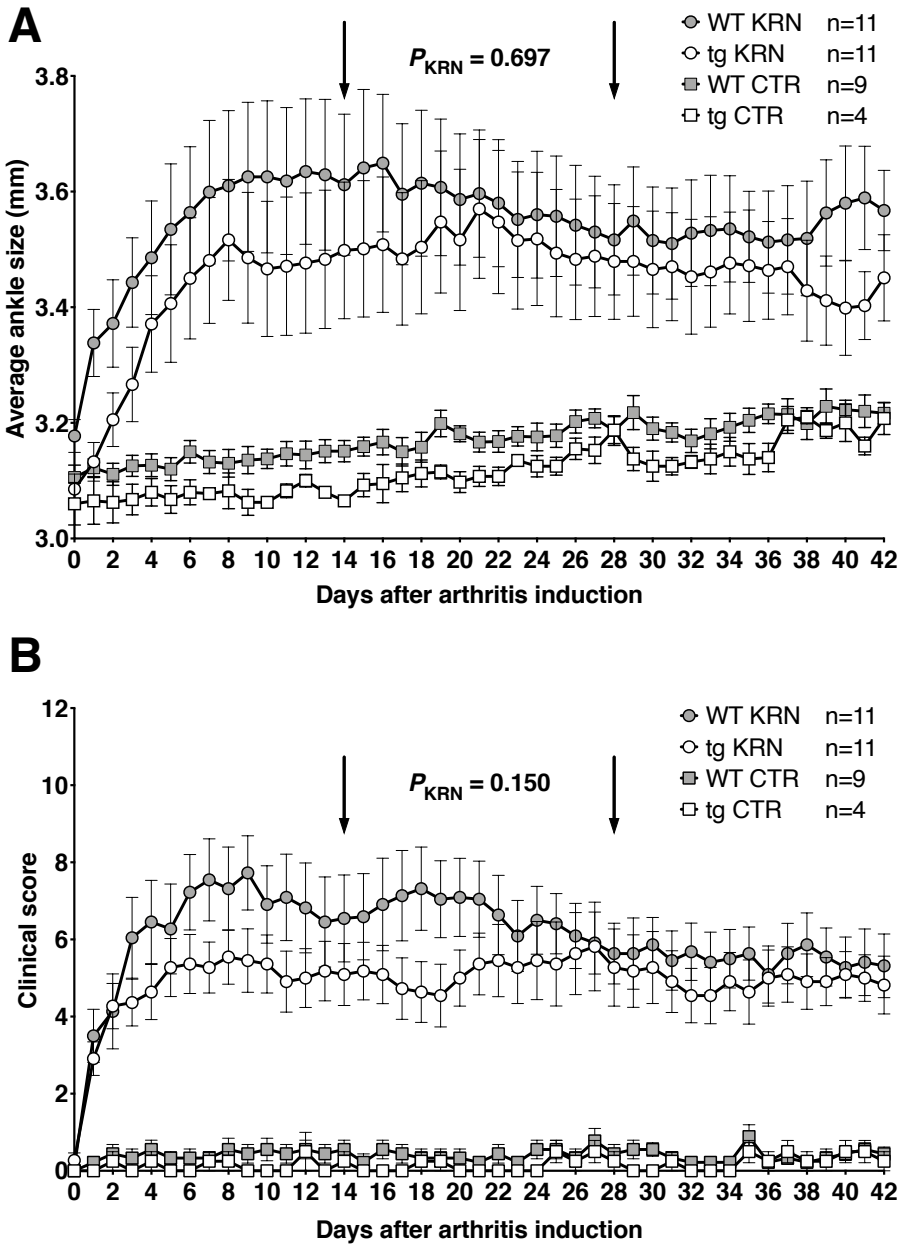

Figure 8: **Clinical course of arthritis over 42 days in the long-term K/BxN serum-transfer arthritis model** After initial induction of arthritis on day 0 and 2, mice received repeated boost injections of arthritogenic KRN-serum (KRN) on days 14 and 28 **(arrows)**, and controls received PBS. **(A)** Ankle size diameter with means ± SEM indicated for each day. **(B)** Clinical score with means and SEM. P_{KRN} value represents the significance after repeated-measures analysis between tg and WT KRN mice.

3.3.4 Histopathologic assessment of inflamed joints

Consistently with clinical findings, histopathologic indices for inflammatory activity were significantly higher in arthritic compared to control mice at day 42 after repeated boost injections (Figure 9). Overall inflammation as quantified by histology was moderate. Applying the Bonferroni correction for multiple comparisons, the difference between tg and WT arthritic did not reach statistical significance.

In terms of cartilage degradation, a similar pattern was observed, with significant differences between arthritic and control mice, but no statistically significant difference between tg KRN and WT KRN mice was detected (Figure 9). Overall, cartilage degradation was rather mild to moderate, despite the prolonged course of arthritis of 42 days, but nevertheless consistent with previously published results.

At day 42, bone erosions were assessed at the arthritic site of the tibio-talar joint. The same pattern was observed, with erosions found mostly in arthritic mice, yet the difference between tg KRN and WT KRN did not reach statistical significance after applying the Bonferroni correction (Figure 9).

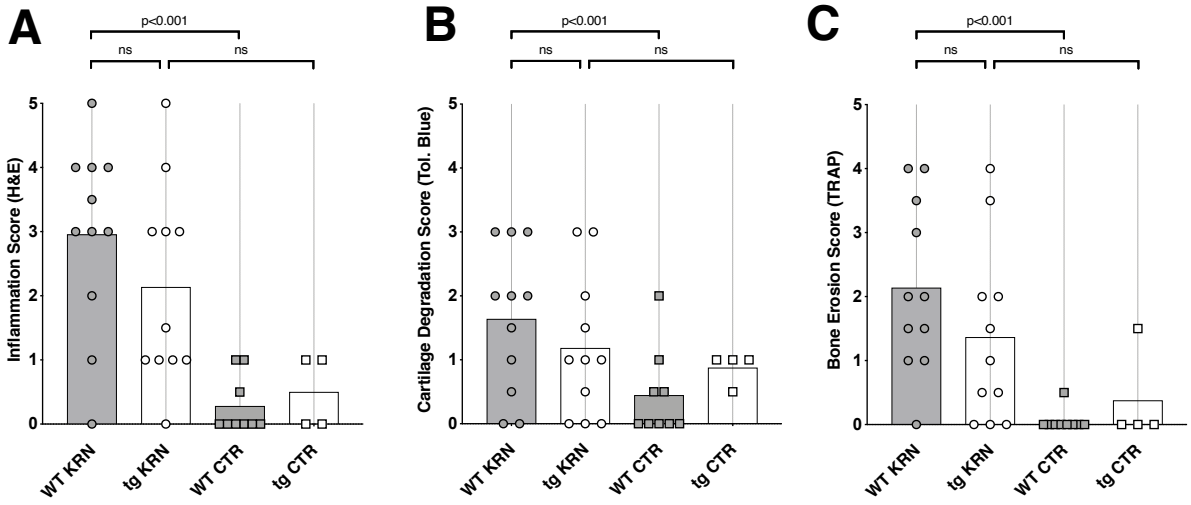

Figure 9: **Histopathologic analysis of ankles after 42 days in the long-term K/BxN serum-transfer arthritis model**

KRN mice received subcutaneous injections of arthritogenic K/BxN serum on days 0 and 2, and further boost injections on days 14 and 28. **(A)** Inflammation Score, **(B)** Cartilage Degradation Score, **(C)** Bone Erosion Score. Scatter dot plot with bars representing mean. ns = not significant. Histological images are not shown.

3.3.5 Micro-CT

Micro-CT analyses were performed to quantify a) the systemic effects of inflammation on bone morphology in a long-term setting and b) to assess local bone morphological changes at the site of arthritis.

In regard to the systemic effects of inflammation on bone, no significant difference was observed for trabecular number, thickness and separation, nor for bone volume over total volume between arthritic and non-arthritic, as well as between tg and WT mice (Figure 11 A, p. 44).

For the analysis of bone erosions, three-dimensional reconstructions of scans were used at the ankle site. A semi-quantitative score was applied in order to measure morphologic changes. Although the overall degree of erosions at the local site was rather mild, WT arthritic mice had significantly more erosive changes than WT controls. This was not observed for transgenic mice, where the degree of erosions at the ankle site did not differ significantly between transgenic arthritic and control mice, possibly suggesting that tg arthritic mice were somewhat protected from the erosions, although the difference between tg KRN and WT KRN was not significant (Figure 10).

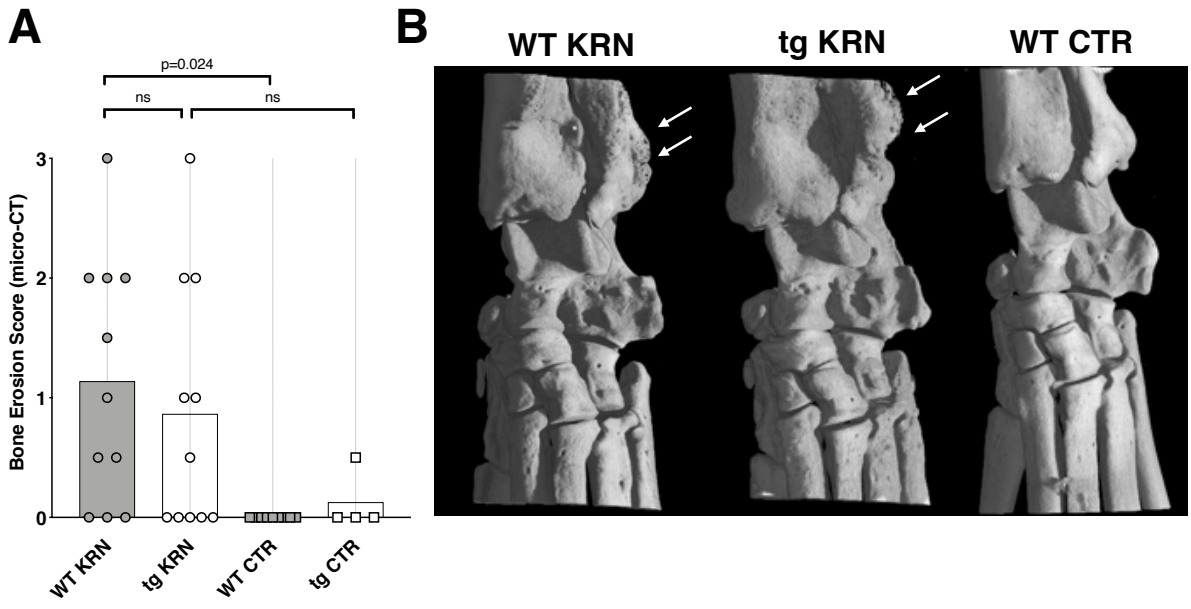

Figure 10: **Assessment of local bone erosions in the long-term K/BxN serum-transfer arthritis experiment by micro-CT at day 42**
KRN mice received arthritogenic K/BxN serum subcutaneously on days 0 and 2, and subsequent boost injections at days 14 and 28 to prolong the course of arthritis. **(A)** Micro-CT based semi-quantitative score for bone erosions: 0=no erosions or cortical damage; 1=mild cortical bone transformation and some erosive changes; 2=moderate bone damage comprising erosions and vacuoles; 3=severe damage with extensive erosions and full-thickness defects of the corticalis. Scatter dot plot with bars representing mean. **(B)** Representative 3D reconstructions of affected ankles. Arrows indicate erosions. ns = not significant.

3.3.6 Bone histomorphometry

In contrast to the acute AIA model, the K/BxN serum-induced arthritis model is characterized by symmetric joint inflammation, driven by a systemic inflammatory process. In order to characterize the long-term systemic effects of inflammation on bone, histomorphometric analysis was performed at the proximal tibia. Despite long-term inflammatory activity, no statistically significant effect of arthritis was detected in bone turnover as measured by mean osteoclast number per bone surface, mean osteoclast surface per bone surface and mean osteoblast surface per bone surface (Figure 11), although WT arthritic mice tended to have slightly higher osteoclast surface and osteoclast number per bone surface compared to controls, consistent with the effects of systemic inflammation on osteoclasts. Numerically higher osteoblast numbers in WT KRN mice in comparison to controls was observed at day 42 – possibly indicating the reactivation of bone formation in response to higher bone turnover. Notably, bone metabolism seemed to be higher overall in transgenic mice compared to WT mice as indicated by numerically higher osteoclast and osteoblast numbers (as was previously described) (162). Inflammatory arthritis did not seem to increase bone turnover in transgenic mice, unlike in WT mice. In fact, there seems to be an arguably reduced bone turnover between tg CTR and tg KRN mice, although tests did not reach statistical significance.

Although serum was collected from all experimental groups, measurements of cytokine levels and bone turnover markers were not performed.

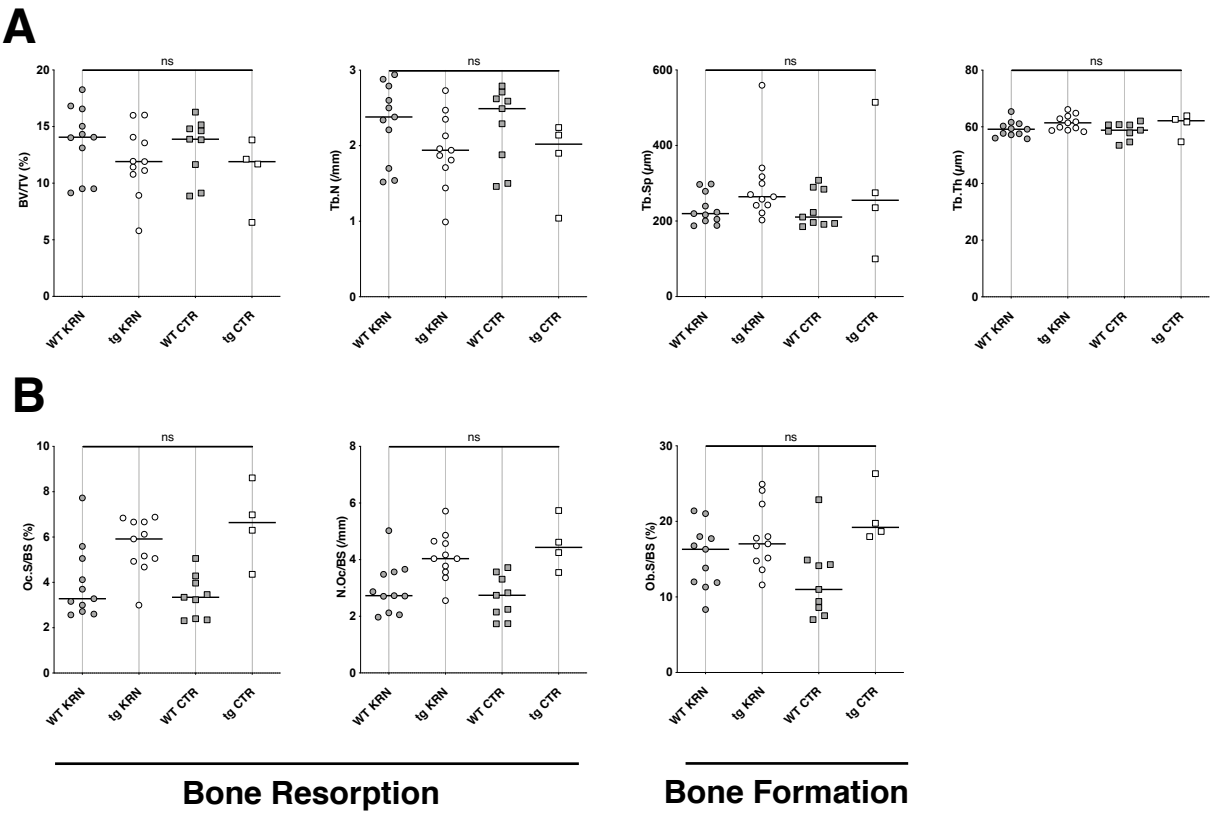

Figure 11: **Bone turnover after 42 days in the long-term K/BxN serum transfer arthritis model**
Bone turnover was measured at the left tibia to assess systemic effects of inflammation on bone. **(A)** Micro-CT. The bone volume fraction (bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th) are shown for bones harvested. Bars show the mean ± SEM. Findings between the 4 groups were compared using Mann-Whitney test with adjustment for 4 parallel tests (α*=0.0125). ns = not significant. **(B)** Histomorphometric quantification. Bone resorption, shown as osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone surface (NOc/BS), respectively. Bone formation, shown as osteoblast surface/bone surface (Ob.S/BS). Scatter dot plot with median. Findings between the 4 groups were compared by Mann-Whitney h adjustment for 4 parallel tests (α*=0.0125). ns = not significant.

4 DISCUSSION

4.1 AIA – a different model to the K/BxN reveals mechanistic properties of immunological features in osteoblasts

Comparing the results from the AIA model with the experiments performed by Buttgereit et al. in 2009, where the disruption of GC signaling in osteoblasts significantly attenuated the course of arthritis in the K/BxN serum-transfer model over a period of 14 days, the current findings clearly stand in stark contrast. Using a relatively large cohort of mice and reaching a degree of arthritis that is comparable to other experiments (128), we have been able to demonstrate that the clinical course is unaffected in terms of knee joint swelling and histology – both being parameters that are well suited for quantification. The same observation can be made for the long-term AIA model, suggesting that the flares, which are caused by re-exposure to the antigen and trigger a T cell driven response, are not mediated by osteoblasts (160).

With the K/BxN arthritis (as published in 2009) clearly being affected by disrupted osteoblastic GC signaling, and the AIA model being virtually not affected, the underlying immunological and inflammatory profiles of both models need to be scrutinized in order to understand the implications of this finding. The juxtaposition of those inflammatory profiles in Table 2 clearly points out essential differences: the AIA model is a T cell-**dependent** model which involves immunological pathways that are complement-**independent**. Immunization leads to an adaptive immune response against a non-self antigen that is later introduced into the knee joints, attracting neutrophils and macrophages. On the other hand, K/BxN serum-induced arthritis is a T cell-independent model where arthritogenic autoantibodies are transferred – resulting in an inflammatory cascade through Fc receptors that requires the innate immune system and relies in particular on the alternative complement pathway. Although the cytokines involved in both models show similarities, the difference lies in particular in the immunological compartments which are mandatory to exert an immunological response.

Table 2: **Immunological and inflammatory profile of AIA and K/BxN serum-transfer arthritis models**
Summarized by Spies, Wiebe et al. (160)

	Antigen-induced Arthritis (AIA)	**K/BxN serum-induced arthritis (KRN)**
T cells	T cell-dependent	T cell-independent
B cells	B cell-independent	B cell-independent
Antibodies	Antibodies not necessary/sufficient to induce arthritis in full extent	Antibodies necessary/sufficient to induce arthritis in full extent
Complement	Complement-independent	Complement-dependent
Fc receptors	FcγR important	FcγR-dependent
Neutrophils, macrophages	Neutrophils important but complement-independent	Neutrophil-dependent, macrophage-dependent
Cytokines	IL-1, TNF-α, IL-6, IL-17, RANKL important	IL-1-, TNF-α-dependent IL-6-, osteopontin-independent

These findings thus lead to the interpretation that osteoblasts modulate inflammatory responses by a GC-dependent pathway that is T cell-independent. From the characteristics of the K/BxN serum-transfer model, it seems plausible that osteoblasts exert their immunomodulatory effects on complement-mediated inflammatory responses in a GC-dependent fashion. This has more recently been corroborated by demonstrating that transgenic disruption of GC signaling in osteoblasts attenuates joint inflammation in yet another model – collagen antibody-induced arthritis (CAIA) – a model similar to the K//BxN serum-transfer model in terms of pathogenesis and underlying inflammatory pathways (163).

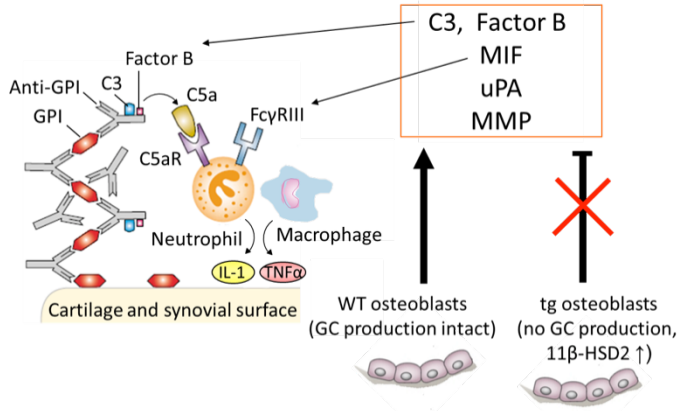

Figure 12: **Hypothesis of a GC-dependent modulation of complement factors by osteoblasts**
Adapted from Leech et al. (164), proposed by Dr. Cornelia Spies. Regulatory factors contribute to local complement activation and its modulation, promoting activation of pro-inflammatory immune cells and leading to local cartilage and bone destruction.

As described in the introduction, the alternative complement pathway seems to stand out. In fact, osteoblasts have been shown to express complement factors like C3, factor B, factor H (165) – and, at least in endothelial cells, their expression has been found to be GC-dependent (166). This aligns with the concept that reduction of complement factors secreted by osteoblasts due to transgenic disruption of GC signaling leads to less severe local inflammation and bone destruction. A schematic model is depicted in Figure 12. It strengthens the case for the immunomodulatory role of osteoblasts in a setting where bone cells and immune cells are tightly interlinked, many facets of which have been proposed and shown since the foundation of the field of osteoimmunology. However, other potential inflammatory factors could also be of possible importance in GC-dependent osteoblast signaling. In the K/BxN serum-transfer model, as well as the CAIA model, i.e., in inflammatory pathways involving immune complex formation and complement activation (167), urokinase-type plasminogen activator (u-PA) has been described as a relevant factor for arthritis progression due to the effects on matrix metalloproteinase (MMP) activation (168, 169). Interestingly, u-PA has also been shown to be upregulated by osteoblasts (170). In contrast, in AIA, u-PA has even been shown to be protective (171, 172). Macrophage migration inhibitory factor (MIF) is also known to be involved in arthritic processes by regulating chemotactic responses of neutrophils (173) in a GC-dependent manner (174) – where low dose GCs paradoxically seem to induce this pro-inflammatory cytokine. Since osteoblasts also express MIF (175), this could be a potential factor by which osteoblasts modulate

inflammation at a local level. However, it has to be acknowledged that MIF is also a relevant factor in AIA arthritis (164), so that its role in the context of the different effects of osteoblastic GC signaling in different arthritis models, needs to be relativized. Although osteoblasts have been described to secrete IL-6 (176), it is less likely to be relevant for the overall modulation of inflammatory processes since in the K/BxN model, IL-6 is of minor importance. The same is likely to apply for IL-1β, which is also expressed by osteoblasts, yet involved in both arthritis models.

The evidence and relevance for the human biological system and in RA in particular still needs to be strengthened. 11β-HSD1 is also expressed in human osteoblasts (105). There is conflicting evidence on the role of GCs when used therapeutically in chronic rheumatic diseases in regard to bone (177) – on the one hand, low-dose GC treatment in RA patients can prevent bone erosions (178), while at the same time, evidence exists that even low doses can have detrimental effects on bone in terms of bone loss (178). Intracellular GC metabolism of osteoblasts and their dependent secretion of pro-inflammatory complement factor might play a significant role in this. It is, however, important to recognize that in arthritis many factors and players are interrelated - for example, synovitis leading to local activation of bone cells and vice versa.

In the multiplex analysis of serum cytokine levels between arthritic and control WT mice no difference was observed at day 14, which is in line with previous studies analyzing 24 cytokines in synovial fluid and sera of rats with AIA at different time points, showing only minor variation in serum, even at inflammation peaks (179). For the local expression of cytokines in the synovial fluid, some differences were observed which correlated to inflammation (129). Since the AIA model is a monoarticular model, cytokine expression is also confined mostly to the inflammation in a single joint, with no measurable increases in the systemic blood stream due to dilution or degradation by the lymphatic system. With that in mind, the difference between tg and WT mice for IL-1a, IL-12, Il-13 after 14 days and its relevance is not quite clear, especially since for the long-term AIA experiment the results were in direct opposition. Although GCs do modulate IL-1a (180, 181), IL-12 (182), and IL-13 (183), the modulation by osteoblasts of these cytokines does not seem to play a crucial role for the course of AIA, as

indicated by the lack of differences between arthritic and control mice. Although we collected synovial fluid and tissues for RNA analysis, both in acute and long-term AIA, we did not analyze the expression of cytokines and genes due to the absent differences between tg and WT mice.

Consistent with serum cytokine expression, and with existing literature, the AIA model does not have systemic effects on bone, as indicated by unchanged bone turnover parameters at the contralateral tibia by micro-CT and histomorphometry. This is not surprising considering the monoarticular nature of the arthritis model, which also remains a local inflammatory reaction after induction of flares by repeated intravenous mBSA injections. With regard to bone erosions at a local level, the degree was rather mild, also for WT mice, and even more so in the long-term model, suggesting that the long-term AIA model, at least when induced by intravenous boosts, would probably not be the preferred model to assess bone changes. This may be different for intra-articular injections, which we did not apply out of concern for local iatrogenic damage which could have confounded the results. However, studies have shown that even after intra-articular re-injections of the antigen, the degree of bone erosions is rather mild, with no difference between acute and long-term experiments, even though the degree of flares was increasing with each set of injections (127, 184). Still, it has to be mentioned that the rate of anaphylactic reactions after intravenous re-injections was relatively high, which was unexpected and not previously described as clearly in literature (severe anaphylactic reactions have only been reported for higher doses of 1000 μg mBSA) (156), so that we emphatically advise against the use of this model for modelling chronic, flaring arthritis.

4.2 The K/BxN long-term model – investigating the impact of endogenous GC signaling in chronic inflammatory processes

In the originally described study by Buttgereit et al., arthritis and joint destruction by transgenic disruption of GC signaling in osteoblasts was significantly attenuated in the K/BxN serum-transfer model (153). While the findings were highly significant in the subscores of total inflammation for synovitis and cartilage, they were less evident for indices of bone erosion. Since the original experiments were conducted over a period

of up to 14 days only, and bone erosions, at least in theory, might take longer to evolve, we decided to prolong the course of arthritis using a long-term model with repeated boost-injections of autoantibodies. This chronic inflammatory setting was also designed to assess the effects of the transgene construct on systemic bone loss.

Arthritis was effectively prolonged over a period of 42 days. However, unlike the previous findings from 2009, the effect of transgenic disruption was less clear for inflammation and bone destruction in the clinical course, as well as in the histological and micro-CT analysis. Applying a conservative statistical analysis of repeated-measures analysis, in which the significant differences in body weight and ankle size at baseline between transgenic and WT mice were controlled for, no statistically significant difference was observed over the entire time course. This suggests that the modulating effect of disrupted GC signaling in osteoblasts in serum-induced autoimmune arthritis seems to abate in a chronic inflammatory setting with prolonged arthritis in contrast to the acute phase, i.e., that the effect of abrogated GC signaling osteoblasts on overall inflammation has less impact over time. Histological assessment and semi-quantification of bone erosions by micro-CT corroborated these findings.

The possible reasons for the findings in this study are multifold and a selection of them will now be discussed briefly.

The K/BxN serum-transfer model elicits arthritis through the transfer of arthritogenic autoantibodies (anti-GPI) contained in the serum of K/BxN mice, which develop arthritis spontaneously. In our experiments, we combined serum of own production with serum that we received from the group of Prof. Wim van den Berg. Although K/BxN serum is considered to be reliably arthritogenic, the titer of anti-GPI antibodies contained in our serum could have possibly been low, which we did not investigate (K/BxN serum producing mice colonies may over time produce less arthritogenic serum) (185, 186). In fact, the K/BxN serum-transfer model has been described as highly sensitive to a threshold concentration of pathogenic antibodies (187). Interestingly, when observing single mice and their course of arthritis, we saw that in WT mice there was a high degree of variability with regard to reactions upon serum

administration (unlike in a retrospective analysis of the data from 2009) – those that reacted upon antibody exposure had severe inflammation in almost all cases, while the others did not show any signs of arthritis, possibly suggesting that a threshold concentration of anti-GPI antibodies in these mice was not reached.
Another aspect is that in the original work by Buttgereit et al., the total number of mice was much higher (28 tg vs 27 WT arthritic mice). In this experiment, we had only 9 tg and 11 WT arthritic mice from many different litters, possibly not statistically powered to show a significant difference between both groups, especially with regard to inflammation until day 14. This is especially relevant when expecting the effect of tg disruption on the course of arthritis by osteoblasts to be mild overall, since many other factors and potent drivers of inflammation, such as the synovium, are also likely to fulfil their role and influence arthritis. Bearing in mind the plethora of cytokines and primary inflammatory cells that are involved in orchestrating autoimmune processes, osteoblasts are unlikely to call the tune.

Looking at the clinical course of arthritis over 42 days as expressed by ankle joint diameter and clinical score, it appears that the effect of transgenic disruption of osteoblastic GC signaling becomes less pronounced over time. Since we did not have the means to measure histological and micro-CT at different time points, to get a more dynamic understanding of the processes involved, the explanation for this can only be speculative. However, understanding that chronic inflammatory processes involve a plethora of physiological mechanisms that drive and halt inflammation, it is plausible that over time physiological anti-inflammatory factors, also present in bone cells, prevail. Another explanation could be that the overall degree of arthritis, although sustained, seems to abate with each re-exposure to autoantibodies (although not described in literature), potentially due to de-sensitization or secretion of neutralizing factors (which we did not investigate).

We used the long-term model of K/BxN serum-induced arthritis in order to focus on bone erosions and systemic bone loss due to chronic inflammation and the effect of transgenic disruption of endogenous GC signaling in osteoblasts. Interestingly, the degree of erosions was rather mild in WT arthritic mice, even after 42 days of persistent

inflammation, and although not directly comparable, overall, it was not more severe than the published experiments from 2009. This might be due to an overall moderate severity of arthritis over the period of 42 days. However, other possible explanations need to be taken into account. Unlike ACPAs, anti-GPI antibodies have not been described to be able to directly induce bone erosions (45).

Systemic bone loss in arthritis was previously observed in the K/BxN model after 14 days, with tg mice being protected against the adverse effect of systemic inflammation on bone metabolism (153). In this set of experiments, tg arthritic mice did not have more increased bone turnover than tg control mice, however, in addition, no systemic effect of inflammation was seen in WT arthritic mice compared to controls. Few studies have analyzed arthritis-induced bone loss over a longer period of time.

Both the relatively mild to moderate degree of erosions and the preserved bone structure could relate to the same mechanisms. It is known that in human RA, repair mechanisms for bone erosions occur after a longer period of time (188, 189) and are usually related to sufficient disease control (190, 191). In RA, the existing disbalance in bone homeostasis is not only a result of inflammation-induced osteoclastogenesis, but also a result of the impaired capacity of osteoblast-lineage cells to form bone (192), resulting in a net bone loss. This is likely to be due to inhibition of Wnt signaling by DKK-1 and secreted Frizzled-related protein (sFRP) families of Wnt signaling antagonists (193). Matzelle et al. showed that in the K/BxN model, when arthritis is allowed to resolve over a long period, significant increase in bone formation is detected due to changes in Wnt signaling (121), which is known to be GC-dependent in osteoblast precursors. Thus, it could well be that in our long-term K/BxN model, repair mechanisms had already set in alongside mild inflammatory conditions, counterbalancing the negative effects on systemic bone and bone erosions, resulting in a net balanced bone turnover, more so in WT mice than in tg mice due to inhibited Wnt signaling by disruption of osteoblastic GC signaling. In fact, the K/BxN serum-transfer model has also shown signs of bone accrual, reminiscent of spondyloarthropathies (144). We did not have the means to apply dynamic histomorphometry to analyze the amount of new bone formation, which would have been helpful to address this important question.

Bearing in mind that GC signaling is also disrupted in osteocytes which have distinct features in the role of bone homeostasis, the relative contribution of osteoblasts and osteocytes cannot be clearly distinguished, yet this might be crucial for understanding the results that have been presented here. Considering the importance of osteocytes in RANKL expression and cell-to-cell communication with osteoclasts, the abating long-term effects and changes could be due to an effect of endogenous GC signaling in osteocytes and their expression of cytokines and other factors (such as sclerostin and DKK-1) in comparison to the effects of GC signaling on osteoblasts, which we did not analyze.

4.3 Future directions

Overall, it seems that it is difficult to model arthritis-induced bone loss over a long period of time by prolonging arthritis in either the AIA model or the K/BxN serum-induced arthritis model. This observation warrants the development of new models to reflect systemic inflammatory bone loss over time, which will be of importance in analyzing the interplay between bone cells and the immune cells in a chronic setting mirroring the chronic inflammatory processes in human affected by rheumatic diseases. For the long-term experiments, different approaches to investigating the effect on bone could be useful for better characterizing the role of chronic inflammation in prolonged animal models, as well as the effect of GC signaling disruption in osteoblasts in these settings. For instance, we did not quantify 'periarticular' bone loss, which could have been achieved by pQCT, allowing us to differentiate between cortical and trabecular bone loss. Equally, immunohistochemistry would be of use in order to analyze the different subsets of inflammatory cells, and potential differences between transgenic and WT mice. Furthermore, we did not quantify the number of osteoclasts in the epiphyseal part of tibia and femur at the arthritic sites, which could have provided further insights. The same is true for characterizing the role of bone marrow cells.

To date, we have not performed RNA analyses of the collected tissues, which may provide insights into gene expression and potential alterations by transgenic disruption of GC signaling.

Future investigations should also be directed at characterizing the role of osteoblasts in driving complement production and how intracellular glucocorticoids affect secretion

of pro-inflammatory cytokines / complement in the osteoblast. New therapies targeted at complement could provide new insights on how this affects bone loss/damage and osteoblasts/osteoclast crosstalk. In vitro studies with osteoblast-osteoclast co-cultures, comparing transgenic to WT osteoblasts, could also be useful to study the gene and cytokine expression, how it affects the complex crosstalk between the both cell types, and how it is affected by other modulation factors.

In conclusion, murine AIA is not affected by disruption of GC signaling in osteoblasts, suggesting that osteoblasts do not modulate T cell-mediated inflammatory responses but rather may impact the alternative complement pathway via a GC-dependent pathway.

This study also advances our knowledge on the GC-dependent role of osteoblasts in the modulation of chronic inflammatory arthritis. In the chronically persistent inflammatory setting of K/BxN serum transfer arthritis, disrupted GC-signaling seems to have a minor impact on inflammation and reparatory processes. These results narrow the role of endogenous GC signaling by osteoblasts down to early inflammatory processes.

5 REFERENCES

1. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature. 2003;423(6937):356-61.

2. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol. 2007;7(6):429-42.

3. Firestein GS, McInnes IB. Immunopathogenesis of Rheumatoid Arthritis. Immunity. 2017;46(2):183-96.

4. McDonald DR, Levy O. 3 - Innate Immunity. In: Rich RR, Fleisher TA, Shearer WT, Schroeder HW, Frew AJ, Weyand CM, editors. Clinical Immunology (Fifth Edition). London: Elsevier; 2019. p. 39-53.e1.

5. Edilova MI, Akram A, Abdul-Sater AA. Innate immunity drives pathogenesis of rheumatoid arthritis. Biomedical Journal. 2020.

6. Holers VM, Banda NK. Complement in the Initiation and Evolution of Rheumatoid Arthritis. Frontiers in Immunology. 2018;9(1057).

7. Trouw LA, Haisma EM, Levarht EWN, van der Woude D, Ioan-Facsinay A, Daha MR, Huizinga TWJ, Toes RE. Anti–cyclic citrullinated peptide antibodies from rheumatoid arthritis patients activate complement via both the classical and alternative pathways. Arthritis & Rheumatism. 2009;60(7):1923-31.

8. Anquetil F, Clavel C, Offer G, Serre G, Sebbag M. IgM and IgA Rheumatoid Factors Purified from Rheumatoid Arthritis Sera Boost the Fc Receptor– and Complement-Dependent Effector Functions of the Disease-Specific Anti–Citrullinated Protein Autoantibodies. The Journal of Immunology. 2015;194(8):3664-74.

9. Erin EW, Paul RT, Lorne JH. Protein Arginine Deiminases and Associated Citrullination: Physiological Functions and Diseases Associated with Dysregulation. Current Drug Targets. 2015;16(7):700-10.

10. Perrin LH, Zubler RH, Nydegger UE, Lambert PH, Miescher PA. Correlation between levels of breakdown products of C3, C4, and properdin factor b in synovial fluids from patients with rheumatoid arthritis. Arthritis & Rheumatism. 1977;20(2):647-52.

11. Ruddy S, Colten HR. Rheumatoid Arthritis. New England Journal of Medicine. 1974;290(23):1284-8.

12. Nakagawa K, Sakiyama H, Tsuchida T, Yamaguchi K, Toyoguchi T, Masuda R, Moriya H. Complement C1s activation in degenerating articular cartilage of rheumatoid arthritis patients: immunohistochemical studies with an active form specific antibody. Annals of the Rheumatic Diseases. 1999;58(3):175-81.

13. Cooke TD, Hurd ER, Jasin HE, Bienenstock J, Ziff M. Identification of immunoglobulins and complement in rheumatoid articular collagenous tissues. Arthritis & Rheumatism. 1975;18(6):541-51.

14. McInnes IB, Buckley CD, Isaacs JD. Cytokines in rheumatoid arthritis — shaping the immunological landscape. Nature Reviews Rheumatology. 2016;12(1):63-8.

15. Saferding V, Blüml S. Innate immunity as the trigger of systemic autoimmune diseases. Journal of autoimmunity. 2020;110:102382.

16. Catrina AI, Ytterberg AJ, Reynisdottir G, Malmström V, Klareskog L. Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. Nature Reviews Rheumatology. 2014;10(11):645-53.

17. van Delft MAM, Huizinga TWJ. An overview of autoantibodies in rheumatoid arthritis. Journal of autoimmunity. 2020;110:102392.

18. Alivernini S, Galeazzi M, Peleg H, Tolusso B, Gremese E, Ferraccioli G, Naparstek Y. Is ACPA positivity the main driver for rheumatoid arthritis treatment? Pros and cons. Autoimmunity Reviews. 2017;16(11):1096-102.

19. Klareskog L, Rönnelid J, Lundberg K, Padyukov L, Alfredsson L. Immunity to Citrullinated Proteins in Rheumatoid Arthritis. Annual Review of Immunology. 2008;26(1):651-75.

20. Calabresi E, Petrelli F, Bonifacio AF, Puxeddu I, Alunno A. One year in review 2018: pathogenesis of rheumatoid arthritis. Clinical and experimental rheumatology. 2018;36(2):175-84.

21. Fox DA. The role of T cells in the immunopathogenesis of rheumatoid arthritis. New perspectives. Arthritis & Rheumatism. 1997;40(4):598-609.

22. Pfeifle R, Rothe T, Ipseiz N, Scherer HU, Culemann S, Harre U, Ackermann JA, Seefried M, Kleyer A, Uderhardt S, Haugg B, Hueber AJ, Daum P, Heidkamp GF, Ge C, Böhm S, Lux A, Schuh W, Magorivska I, Nandakumar KS, Lönnblom E, Becker C, Dudziak D, Wuhrer M, Rombouts Y, Koeleman CA, Toes R, Winkler TH, Holmdahl R, Herrmann M, Blüml S, Nimmerjahn F, Schett G, Krönke G. Regulation of autoantibody activity by the IL-23–TH17 axis determines the onset of autoimmune disease. Nature Immunology. 2017;18(1):104-13.

23. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. Current Opinion in Immunology. 2007;19(3):281-6.

24. van Oosterhout M, Bajema I, Levarht EWN, Toes REM, Huizinga TWJ, van Laar JM. Differences in synovial tissue infiltrates between anti–cyclic citrullinated peptide–positive rheumatoid arthritis and anti–cyclic citrullinated peptide–negative rheumatoid arthritis. Arthritis & Rheumatism. 2008;58(1):53-60.

25. Fassbender HG, Seibel M, Hebert T. Pathways of Destruction in Metacarpal and Metatarsal Joints of Patients with Rheumatoid Arthritis. Scandinavian Journal of Rheumatology. 1992;21(1):10-6.

26. Tolboom TCA, van der Helm-Van Mil AHM, Nelissen RGHH, Breedveld FC, Toes REM, Huizinga TWJ. Invasiveness of fibroblast-like synoviocytes is an individual patient characteristic associated with the rate of joint destruction in patients with rheumatoid arthritis. Arthritis & Rheumatism. 2005;52(7):1999-2002.

27. Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid arthritis. The Lancet. 2010;376(9746):1094-108.

28. Schett G. Autoimmunity as a trigger for structural bone damage in rheumatoid arthritis. Mod Rheumatol. 2017;27(2):193-7.

29. Ødegård S, Landewé R, van der Heijde D, Kvien TK, Mowinckel P, Uhlig T. Association of early radiographic damage with impaired physical function in rheumatoid arthritis: A ten-year, longitudinal observational study in 238 patients. Arthritis & Rheumatism. 2006;54(1):68-75.

30. Boissier M-C, Semerano L, Challal S, Saidenberg-Kermanac'h N, Falgarone G. Rheumatoid arthritis: From autoimmunity to synovitis and joint destruction. Journal of autoimmunity. 2012;39(3):222-8.

31. Sharp JT, Wolfe F, Mitchell DM, Bloch DA. The progression of erosion and joint space narrowing scores in rheumatoid arthritis during the first twenty-five years of disease. Arthritis & Rheumatism. 1991;34(6):660-8.

32. Schett G, Gravallese E. Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. Nat Rev Rheumatol. 2012;8(11):656-64.

33. van Staa TP, Geusens P, Bijlsma JW, Leufkens HG, Cooper C. Clinical assessment of the long-term risk of fracture in patients with rheumatoid arthritis. Arthritis and rheumatism. 2006;54(10):3104-12.

34. Hooyman JR, Melton LJ, 3rd, Nelson AM, O'Fallon WM, Riggs BL. Fractures after rheumatoid arthritis. A population-based study. Arthritis and rheumatism. 1984;27(12):1353-61.

35. Kim SY, Schneeweiss S, Liu J, Daniel GW, Chang CL, Garneau K, Solomon DH. Risk of osteoporotic fracture in a large population-based cohort of patients with rheumatoid arthritis. Arthritis Res Ther. 2010;12(4):R154.

36. Wright NC, Lisse JR, Walitt BT, Eaton CB, Chen Z, Women's Health Initiative I. Arthritis increases the risk for fractures--results from the Women's Health Initiative. J Rheumatol. 2011;38(8):1680-8.

37. Briot K, Geusens P, Em Bultink I, Lems WF, Roux C. Inflammatory diseases and bone fragility. Osteoporos Int. 2017;28(12):3301-14.

38. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nature Reviews Immunology. 2007;7(4):292-304.

39. McLean RR. Proinflammatory cytokines and osteoporosis. Curr Osteoporos Rep. 2009;7(4):134-9.

40. Goldring SR, Gravallese EM. Pathogenesis of bone erosions in rheumatoid arthritis. Current Opinion in Rheumatology. 2000;12(3):195-9.

41. Abu-Amer Y. NF-kappaB signaling and bone resorption. Osteoporos Int. 2013;24(9):2377-86.

42. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T. Commitment and Differentiation of Osteoclast Precursor Cells by the Sequential Expression of C-Fms and Receptor Activator of Nuclear Factor κb (Rank) Receptors. Journal of Experimental Medicine. 1999;190(12):1741-54.

43. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T. Tumor Necrosis Factor α Stimulates Osteoclast Differentiation by a

Mechanism Independent of the Odf/Rankl–Rank Interaction. Journal of Experimental Medicine. 2000;191(2):275-86.

44. Axmann R, Herman S, Zaiss M, Franz S, Polzer K, Zwerina J, Herrmann M, Smolen J, Schett G. CTLA-4 directly inhibits osteoclast formation. Ann Rheum Dis. 2008;67(11):1603-9.

45. Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, Jakobsson PJ, Baum W, Nimmerjahn F, Szarka E, Sarmay G, Krumbholz G, Neumann E, Toes R, Scherer HU, Catrina AI, Klareskog L, Jurdic P, Schett G. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. J Clin Invest. 2012;122(5):1791-802.

46. Steffen U, Schett G, Bozec A. How Autoantibodies Regulate Osteoclast Induced Bone Loss in Rheumatoid Arthritis. Frontiers in Immunology. 2019;10(1483).

47. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT, Martin TJ, Suda T. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. The Journal of Clinical Investigation. 1999;103(9):1345-52.

48. Ogata Y, Kukita A, Kukita T, Komine M, Miyahara A, Miyazaki S, Kohashi O. A Novel Role of IL-15 in the Development of Osteoclasts: Inability to Replace Its Activity with IL-2. The Journal of Immunology. 1999;162(5):2754-60.

49. Terauchi M, Li JY, Bedi B, Baek KH, Tawfeek H, Galley S, Gilbert L, Nanes MS, Zayzafoon M, Guldberg R, Lamar DL, Singer MA, Lane TF, Kronenberg HM, Weitzmann MN, Pacifici R. T lymphocytes amplify the anabolic activity of parathyroid hormone through Wnt10b signaling. Cell Metab. 2009;10(3):229-40.

50. Li Y, Toraldo G, Li A, Yang X, Zhang H, Qian WP, Weitzmann MN. B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. Blood. 2007;109(9):3839-48.

51. Li J-Y, Tawfeek H, Bedi B, Yang X, Adams J, Gao KY, Zayzafoon M, Weitzmann MN, Pacifici R. Ovariectomy disregulates osteoblast and osteoclast formation through the T-cell receptor CD40 ligand. Proceedings of the National Academy of Sciences. 2011;108(2):768.

52. Teitelbaum SL. Bone resorption by osteoclasts. Science. 2000;289(5484):1504-8.

53. Matsuo K, Irie N. Osteoclast-osteoblast communication. Arch Biochem Biophys. 2008;473(2):201-9.

54. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiki S, Matsuda T, Hirano T, Kishimoto T, Suda T. Il-6 Is Produced by Osteoblasts and Induces Bone-Resorption. Journal of Immunology. 1990;145(10):3297-303.

55. Ignatius A, Schoengraf P, Kreja L, Liedert A, Recknagel S, Kandert S, Brenner RE, Schneider M, Lambris JD, Huber-Lang M. Complement C3a and C5a modulate osteoclast formation and inflammatory response of osteoblasts in synergism with IL-1beta. J Cell Biochem. 2011;112(9):2594-605.

56. Palmowski Y, Buttgereit T, Buttgereit F. The 70th anniversary of glucocorticoids in rheumatic diseases: the second youth of an old friend. Rheumatology. 2018;58(4):580-7.

57. Saldanha C, Tougas G, Grace E. Evidence for anti-inflammatory effect of normal circulating plasma cortisol. Clinical and experimental rheumatology. 1986;4(4):365-6.

58. Hench PS, Kendall EC. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Proceedings of the staff meetings Mayo Clinic. 1949;24(8):181-97.

59. Alten R, Wiebe E. Hypothalamic-pituitary-adrenal axis function in patients with rheumatoid arthritis treated with different glucocorticoid approaches. Neuroimmunomodulation. 2015;22(1-2):83-8.

60. Hoes JN, Jacobs JWG, Boers M, Boumpas D, Buttgereit F, Caeyers N, Choy EH, Cutolo M, Da Silva JAP, Esselens G, Guillevin L, Hafstrom I, Kirwan JR, Rovensky J, Russell A, Saag KG, Svensson B, Westhovens R, Zeidler H, Bijlsma JWJ. EULAR evidence-based recommendations on the management of systemic glucocorticoid therapy in rheumatic diseases. Annals of the Rheumatic Diseases. 2007;66(12):1560-7.

61. Smolen JS, Landewé R, Bijlsma J, Burmester G, Chatzidionysiou K, Dougados M, Nam J, Ramiro S, Voshaar M, van Vollenhoven R, Aletaha D, Aringer M, Boers M, Buckley CD, Buttgereit F, Bykerk V, Cardiel M, Combe B, Cutolo M, van Eijk-Hustings Y, Emery P, Finckh A, Gabay C, Gomez-Reino J, Gossec L, Gottenberg J-E, Hazes JMW, Huizinga T, Jani M, Karateev D, Kouloumas M, Kvien T, Li Z, Mariette X, McInnes I, Mysler E, Nash P, Pavelka K, Poór G, Richez C, van Riel P, Rubbert-Roth A, Saag K, da Silva J, Stamm T, Takeuchi T, Westhovens R, de Wit M, van der Heijde D. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2016 update. Annals of the Rheumatic Diseases. 2017;76(6):960-77.

62. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. Nature Reviews Immunology. 2017;17(4):233-47.

63. Jacobs JW, van Everdingen AA, Verstappen SM, Bijlsma JW. Followup radiographic data on patients with rheumatoid arthritis who participated in a two-year trial of prednisone therapy or placebo. Arthritis and rheumatism. 2006;54(5):1422-8.

64. Wassenberg S, Rau R, Steinfeld P, Zeidler H. Very low-dose prednisolone in early rheumatoid arthritis retards radiographic progression over two years: a multicenter, double-blind, placebo-controlled trial. Arthritis and rheumatism. 2005;52(11):3371-80.

65. Svensson B, Boonen A, Albertsson K, van der Heijde D, Keller C, Hafström I. Low-dose prednisolone in addition to the initial disease-modifying antirheumatic drug in patients with early active rheumatoid arthritis reduces joint destruction and increases the remission rate: a two-year randomized trial. Arthritis and rheumatism. 2005;52(11):3360-70.

66. Van Staa TP, Leufkens HG, Abenhaim L, Zhang B, Cooper C. Use of oral corticosteroids and risk of fractures. June, 2000. J Bone Miner Res. 2005;20(8):1487-94; discussion 6.

67. Walsh LJ, Lewis SA, Wong CA, Cooper S, Oborne J, Cawte SA, Harrison T, Green DJ, Pringle M, Hubbard R, Tattersfield AE. The impact of oral corticosteroid use on bone mineral density and vertebral fracture. Am J Respir Crit Care Med. 2002;166(5):691-5.

68. Hardy RS, Zhou H, Seibel MJ, Cooper MS. Glucocorticoids and Bone: Consequences of Endogenous and Exogenous Excess and Replacement Therapy. Endocr Rev. 2018;39(5):519-48.

69. Hardy R, Cooper MS. Glucocorticoid-induced osteoporosis - a disorder of mesenchymal stromal cells? Front Endocrinol (Lausanne). 2011;2(24):24.

70. Bennett A, Chen T, Feldman D, Hintz RL, Rosenfeld RG. Characterization of insulin-like growth factor I receptors on cultured rat bone cells: regulation of receptor concentration by glucocorticoids. Endocrinology. 1984;115(4):1577-83.

71. Centrella M, McCarthy TL, Canalis E. Glucocorticoid regulation of transforming growth factor beta 1 activity and binding in osteoblast-enriched cultures from fetal rat bone. Molecular and cellular biology. 1991;11(9):4490-6.

72. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids - Potential mechanisms of their deleterious effects on bone. Journal of Clinical Investigation. 1998;102(2):274-82.

73. Weinstein RS. Glucocorticoid-induced osteonecrosis. Endocrine. 2012;41(2):183-90.

74. Jia J, Yao W, Guan M, Dai W, Shahnazari M, Kar R, Bonewald L, Jiang JX, Lane NE. Glucocorticoid dose determines osteocyte cell fate. FASEB J. 2011;25(10):3366-76.

75. Walsh MC, Choi Y. Biology of the RANKL–RANK–OPG System in Immunity, Bone, and Beyond. Frontiers in Immunology. 2014;5(511).

76. Swanson C, Lorentzon M, Conaway HH, Lerner UH. Glucocorticoid Regulation of Osteoclast Differentiation and Expression of Receptor Activator of Nuclear Factor-κB (NF-κB) Ligand, Osteoprotegerin, and Receptor Activator of NF-κB in Mouse Calvarial Bones. Endocrinology. 2006;147(7):3613-22.

77. Ohnaka K, Taniguchi H, Kawate H, Nawata H, Takayanagi R. Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis. Biochemical and Biophysical Research Communications. 2004;318(1):259-64.

78. Yao W, Cheng Z, Busse C, Pham A, Nakamura MC, Lane NE. Glucocorticoid excess in mice results in early activation of osteoclastogenesis and adipogenesis and prolonged suppression of osteogenesis: A longitudinal study of gene expression in bone tissue from glucocorticoid-treated mice. Arthritis & Rheumatism. 2008;58(6):1674-86.

79. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. Oncogene. 2006;25(57):7469-81.

80. Hardy RS, Raza K, Cooper MS. Therapeutic glucocorticoids: mechanisms of actions in rheumatic diseases. Nature Reviews Rheumatology. 2020;16(3):133-44.

81. Brewer JA, Khor B, Vogt SK, Muglia LM, Fujiwara H, Haegele KE, Sleckman BP, Muglia LJ. T-cell glucocorticoid receptor is required to suppress COX-2-mediated lethal immune activation. Nat Med. 2003;9(10):1318-22.

82. Son GH, Cha HK, Chung S, Kim K. Multimodal Regulation of Circadian Glucocorticoid Rhythm by Central and Adrenal Clocks. J Endocr Soc. 2018;2(5):444-59.

83. Spies CM, Straub RH, Cutolo M, Buttgereit F. Circadian rhythms in rheumatology--a glucocorticoid perspective. Arthritis Res Ther. 2014;16 Suppl 2(2):S3.

84. Manenschijn L, van den Akker EL, Lamberts SW, van Rossum EF. Clinical features associated with glucocorticoid receptor polymorphisms. An overview. Ann N Y Acad Sci. 2009;1179(1):179-98.

85. Buttgereit F, Straub RH, Wehling M, Burmester GR. Glucocorticoids in the treatment of rheumatic diseases: an update on the mechanisms of action. Arthritis and rheumatism. 2004;50(11):3408-17.

86. Almawi WY, Melemedjian OK. Negative regulation of nuclear factor-kappaB activation and function by glucocorticoids. Journal of molecular endocrinology. 2002;28(2):69-78.

87. Buttgereit F, Burmester GR, Straub RH, Seibel MJ, Zhou H. Exogenous and endogenous glucocorticoids in rheumatic diseases. Arthritis and rheumatism. 2011;63(1):1-9.

88. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol. 1994;10(1):405-55.

89. Stahn C, Buttgereit F. Genomic and nongenomic effects of glucocorticoids. Nature clinical practice Rheumatology. 2008;4(10):525-33.

90. Schacke H, Rehwinkel H, Asadullah K, Cato AC. Insight into the molecular mechanisms of glucocorticoid receptor action promotes identification of novel ligands with an improved therapeutic index. Experimental dermatology. 2006;15(8):565-73.

91. Falkenstein E, Norman AW, Wehling M. Mannheim classification of nongenomically initiated (rapid) steroid action(s). The Journal of clinical endocrinology and metabolism. 2000;85(5):2072-5.

92. Buttgereit F, Wehling M, Burmester G-Rd. A new hypothesis of modular glucocorticoid actions: Steroid treatment of rheumatic diseases revisited. Arthritis & Rheumatism. 1998;41(5):761-7.

93. Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester GR, Lauster R, Scheffold A, Buttgereit F. Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. FASEB J. 2004;18(1):70-80.

94. Lowenberg M, Verhaar AP, Bilderbeek J, Marle J, Buttgereit F, Peppelenbosch MP, van Deventer SJ, Hommes DW. Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN. EMBO Rep. 2006;7(10):1023-9.

95. Baschant U, Frappart L, Rauchhaus U, Bruns L, Reichardt HM, Kamradt T, Bräuer R, Tuckermann JP. Glucocorticoid therapy of antigen-induced arthritis depends on the dimerized glucocorticoid receptor in T cells. Proc Natl Acad Sci U S A. 2011;108(48):19317-22.

96. Gomez-Sanchez E, Gomez-Sanchez CE. The multifaceted mineralocorticoid receptor. Comprehensive Physiology. 2014;4(3):965-94.

97. Mercer WR, Krozowski ZS. Localization of an 11 beta hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. Endocrinology. 1992;130(1):540-3.

98. Smith RE, Maguire JA, Stein-Oakley AN, Sasano H, Takahashi K, Fukushima K, Krozowski ZS. Localization of 11 beta-hydroxysteroid dehydrogenase type II in human epithelial tissues. The Journal of clinical endocrinology and metabolism. 1996;81(9):3244-8.

99. Roland BL, Funder JW. Localization of 11beta-hydroxysteroid dehydrogenase type 2 in rat tissues: in situ studies. Endocrinology. 1996;137(3):1123-8.

100. Tannin GM, Agarwal AK, Monder C, New MI, White PC. The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. J Biol Chem. 1991;266(25):16653-8.

101. Neeck G, Federlin K, Graef V, Rusch D, Schmidt KL. Adrenal secretion of cortisol in patients with rheumatoid arthritis. J Rheumatol. 1990;17(1):24-9.

102. Gudbjörnsson B, Skogseid B, Oberg K, Wide L, Hällgren R. Intact adrenocorticotropic hormone secretion but impaired cortisol response in patients with active rheumatoid arthritis. Effect of glucocorticoids. J Rheumatol. 1996;23(4):596-602.

103. Gilmour JS, Coutinho AE, Cailhier J-F, Man TY, Clay M, Thomas G, Harris HJ, Mullins JJ, Seckl JR, Savill JS, Chapman KE. Local Amplification of Glucocorticoids by 11β-Hydroxysteroid Dehydrogenase Type 1 Promotes Macrophage Phagocytosis of Apoptotic Leukocytes. The Journal of Immunology. 2006;176(12):7605-11.

104. Tu J, Stoner S, Fromm PD, Wang T, Chen D, Tuckermann J, Cooper MS, Seibel MJ, Zhou H. Endogenous glucocorticoid signaling in chondrocytes attenuates joint inflammation and damage. Faseb j. 2018;32(1):478-87.

105. Cooper MS, Walker EA, Bland R, Fraser WD, Hewison M, Stewart PM. Expression and functional consequences of 11β-hydroxysteroid dehydrogenase activity in human bone. Bone. 2000;27(3):375-81.

106. Fenton C, Martin C, Jones R, Croft A, Campos J, Naylor AJ, Taylor AE, Chimen M, Cooper M, Lavery GG, Raza K, Hardy RS. Local steroid activation is a critical mediator of the anti-inflammatory actions of therapeutic glucocorticoids. Annals of the Rheumatic Diseases. 2020:annrheumdis-2020-218493.

107. Hardy R, Rabbitt EH, Filer A, Emery P, Hewison M, Stewart PM, Gittoes NJ, Buckley CD, Raza K, Cooper MS. Local and systemic glucocorticoid metabolism in inflammatory arthritis. Annals of the Rheumatic Diseases. 2008;67(9):1204-10.

108. Hardy RS, Filer A, Cooper MS, Parsonage G, Raza K, Hardie DL, Rabbitt EH, Stewart PM, Buckley CD, Hewison M. Differential expression, function and response to inflammatory stimuli of 11beta-hydroxysteroid dehydrogenase type 1 in human fibroblasts: a mechanism for tissue-specific regulation of inflammation. Arthritis Res Ther. 2006;8(4):R108.

109. Hardy RS, Fenton C, Croft AP, Naylor AJ, Begum R, Desanti G, Buckley CD, Lavery G, Cooper MS, Raza K. 11 Beta-hydroxysteroid dehydrogenase type 1 regulates synovitis, joint destruction, and systemic bone loss in chronic polyarthritis. Journal of autoimmunity. 2018;92:104-13.

110. Coutinho AE, Gray M, Brownstein DG, Salter DM, Sawatzky DA, Clay S, Gilmour JS, Seckl JR, Savill JS, Chapman KE. 11β-Hydroxysteroid Dehydrogenase Type 1, But Not Type 2, Deficiency Worsens Acute Inflammation and Experimental Arthritis in Mice. Endocrinology. 2012;153(1):234-40.

111. Schmidt M, Weidler C, Naumann H, Anders S, Schölmerich J, Straub RH. Reduced capacity for the reactivation of glucocorticoids in rheumatoid arthritis synovial cells: possible role of the sympathetic nervous system? Arthritis and rheumatism. 2005;52(6):1711-20.

112. Haas CS, Creighton CJ, Pi X, Maine I, Koch AE, Haines GK, Ling S, Chinnaiyan AM, Holoshitz J. Identification of genes modulated in rheumatoid arthritis using complementary DNA microarray analysis of lymphoblastoid B cell lines from disease-discordant monozygotic twins. Arthritis and rheumatism. 2006;54(7):2047-60.

113. Cooper MS, Walker EA, Bland R, Fraser WD, Hewison M, Stewart PM. Expression and functional consequences of 11beta-hydroxysteroid dehydrogenase activity in human bone. Bone. 2000;27(3):375-81.

114. Bland R, Worker CA, Noble BS, Eyre LJ, Bujalska IJ, Sheppard MC, Stewart PM, Hewison M. Characterization of 11beta-hydroxysteroid dehydrogenase activity and corticosteroid receptor expression in human osteosarcoma cell lines. J Endocrinol. 1999;161(3):455-64.

115. Cooper MS, Rabbitt EH, Goddard PE, Bartlett WA, Hewison M, Stewart PM. Osteoblastic 11beta-hydroxysteroid dehydrogenase type 1 activity increases with age and glucocorticoid exposure. J Bone Miner Res. 2002;17(6):979-86.

116. Condon J, Gosden C, Gardener D, Nickson P, Hewison M, Howie AJ, Stewart PM. Expression of type 2 11beta-hydroxysteroid dehydrogenase and corticosteroid hormone receptors in early human fetal life. The Journal of clinical endocrinology and metabolism. 1998;83(12):4490-7.

117. Henneicke H, Herrmann M, Kalak R, Brennan-Speranza TC, Heinevetter U, Bertollo N, Day RE, Huscher D, Buttgereit F, Dunstan CR, Seibel MJ, Zhou H. Corticosterone selectively targets endo-cortical surfaces by an osteoblast-dependent mechanism. Bone. 2011;49(4):733-42.

118. Sher LB, Woitge HW, Adams DJ, Gronowicz GA, Krozowski Z, Harrison JR, Kream BE. Transgenic expression of 11beta-hydroxysteroid dehydrogenase type 2 in osteoblasts reveals an anabolic role for endogenous glucocorticoids in bone. Endocrinology. 2004;145(2):922-9.

119. Kalak R, Zhou H, Street J, Day RE, Modzelewski JR, Spies CM, Liu PY, Li G, Dunstan CR, Seibel MJ. Endogenous glucocorticoid signalling in osteoblasts is necessary to maintain normal bone structure in mice. Bone. 2009;45(1):61-7.

120. Zhou H, Mak W, Zheng Y, Dunstan CR, Seibel MJ. Osteoblasts directly control lineage commitment of mesenchymal progenitor cells through Wnt signaling. J Biol Chem. 2008;283(4):1936-45.

121. Zhou H, Mak W, Kalak R, Street J, Fong-Yee C, Zheng Y, Dunstan CR, Seibel MJ. Glucocorticoid-dependent Wnt signaling by mature osteoblasts is a key regulator of cranial skeletal development in mice. Development. 2009;136(3):427-36.

122. Diarra D, Stolina M, Polzer K, Zwerina J, Ominsky MS, Dwyer D, Korb A, Smolen J, Hoffmann M, Scheinecker C, van der Heide D, Landewe R, Lacey D, Richards WG, Schett G. Dickkopf-1 is a master regulator of joint remodeling. Nature Medicine. 2007;13(2):156-63.

123. Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, Kirilov M, Mandic V, Takacz A, Schmidt-Ullrich R, Ostermay S, Schinke T, Spanbroek R, Zaiss MM, Angel PE, Lerner UH, David JP, Reichardt HM, Amling M, Schutz G, Tuckermann JP. Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. Cell Metab. 2010;11(6):517-31.

124. Mak W, Shao X, Dunstan CR, Seibel MJ, Zhou H. Biphasic glucocorticoid-dependent regulation of Wnt expression and its inhibitors in mature osteoblastic cells. Calcif Tissue Int. 2009;85(6):538-45.

125. Brackertz D, Mitchell GF, Mackay IR. Antigen-induced arthritis in mice. I. Induction of arthritis in various strains of mice. Arthritis and rheumatism. 1977;20(3):841-50.

126. Wong PK, Quinn JM, Sims NA, van Nieuwenhuijze A, Campbell IK, Wicks IP. Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis. Arthritis and rheumatism. 2006;54(1):158-68.

127. Ebbinghaus M, Gajda M, Boettger MK, Schaible HG, Brauer R. The anti-inflammatory effects of sympathectomy in murine antigen-induced arthritis are associated with a reduction of Th1 and Th17 responses. Ann Rheum Dis. 2012;71(2):253-61.

128. Baschant U, Frappart L, Rauchhaus U, Bruns L, Reichardt HM, Kamradt T, Brauer R, Tuckermann JP. Glucocorticoid therapy of antigen-induced arthritis depends on the dimerized glucocorticoid receptor in T cells. Proc Natl Acad Sci U S A. 2011;108(48):19317-22.

129. Paquet J, Goebel JC, Delaunay C, Pinzano A, Grossin L, Cournil-Henrionnet C, Gillet P, Netter P, Jouzeau JY, Moulin D. Cytokines profiling by multiplex analysis in

experimental arthritis: which pathophysiological relevance for articular versus systemic mediators? Arthritis Res Ther. 2012;14(2):R60.

130. Stenzler SA, Miller F. Failure of cobra venom factor to inhibit antigen-induced arthritis. Arthritis and rheumatism. 1977;20(8):1567.

131. Kourounakis L, Nelson RA, Jr., Kupusta MA. The effect of a cobra venom factor on complement and adjuvant-induced disease in rats. Arthritis and rheumatism. 1973;16(1):71-6.

132. Woodruff TM, Strachan AJ, Dryburgh N, Shiels IA, Reid RC, Fairlie DP, Taylor SM. Antiarthritic activity of an orally active C5a receptor antagonist against antigen-induced monarticular arthritis in the rat. Arthritis and rheumatism. 2002;46(9):2476-85.

133. Macor P, Durigutto P, De Maso L, Garrovo C, Biffi S, Cortini A, Fischetti F, Sblattero D, Pitzalis C, Marzari R, Tedesco F. Treatment of experimental arthritis by targeting synovial endothelium with a neutralizing recombinant antibody to C5. Arthritis and rheumatism. 2012;64(8):2559-67.

134. Durigutto P, Macor P, Ziller F, De Maso L, Fischetti F, Marzari R, Sblattero D, Tedesco F. Prevention of arthritis by locally synthesized recombinant antibody neutralizing complement component C5. PLoS One. 2013;8(3):e58696.

135. Engdahl C, Lindholm C, Stubelius A, Ohlsson C, Carlsten H, Lagerquist MK. Periarticular bone loss in antigen-induced arthritis. Arthritis and rheumatism. 2013;65(11):2857-65.

136. Consden R, Doble A, Glynn LE, Nind AP. Production of a chronic arthritis with ovalbumin. Its retention in the rabbit knee joint. Ann Rheum Dis. 1971;30(3):307-15.

137. van Beusekom HJ, van de Putte LB, van den Berg WB, van den Broek WJ, Buijs WC. Antigen handling in antigen-induced joint inflammation: kinetics of a second intra-articularly injected dose of antigen in an already established antigen-induced joint inflammation. Immunology. 1981;44(1):153-61.

138. Zvaifler NJ. The immunopathology of joint inflammation in rheumatoid arthritis. Advances in immunology. 1973;16(0):265-336.

139. Buchner E, Brauer R, Schmidt C, Emmrich F, Kinne RW. Induction of flare-up reactions in rat antigen-induced arthritis. Journal of autoimmunity. 1995;8(1):61-74.

140. van de Putte LB, Lens JW, van den Berg WB, Kruijsen MW. Exacerbation of antigen-induced arthritis after challenge with intravenous antigen. Immunology. 1983;49(1):161-7.

141. Lens JW, van den Berg WB, van de Putte LB, Zwarts WA. Flare of antigen-induced arthritis in mice after intravenous challenge. Kinetics of antigen in the circulation and localization of antigen in the arthritic and noninflamed joint. Arthritis and rheumatism. 1986;29(5):665-74.

142. van de Loo AA, Arntz OJ, Bakker AC, van Lent PL, Jacobs MJ, van den Berg WB. Role of interleukin 1 in antigen-induced exacerbations of murine arthritis. Am J Pathol. 1995;146(1):239-49.

143. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. Cell. 1996;87(5):811-22.

144. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, Degott C, Kikutani H, Rajewsky K, Pasquali JL, Benoist C, Mathis D. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity. 1999;10(4):451-61.

145. Kyburz D, Corr M. The KRN mouse model of inflammatory arthritis. Springer Semin Immunopathol. 2003;25(1):79-90.

146. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, Gravallese E, Mathis D, Benoist C. Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. J Exp Med. 2002;196(1):77-85.

147. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, Takahashi K, Holers VM, Walport M, Gerard C, Ezekowitz A, Carroll MC, Brenner M, Weissleder R, Verbeek JS, Duchatelle V, Degott C, Benoist C, Mathis D. Arthritis critically dependent on innate immune system players. Immunity. 2002;16(2):157-68.

148. Mancardi DA, Jonsson F, Iannascoli B, Khun H, Van Rooijen N, Huerre M, Daeron M, Bruhns P. Cutting Edge: The murine high-affinity IgG receptor FcgammaRIV is sufficient for autoantibody-induced arthritis. J Immunol. 2011;186(4):1899-903.

149. Monach PA, Nigrovic PA, Chen M, Hock H, Lee DM, Benoist C, Mathis D. Neutrophils in a mouse model of autoantibody-mediated arthritis: critical producers of Fc receptor gamma, the receptor for C5a, and lymphocyte function-associated antigen 1. Arthritis and rheumatism. 2010;62(3):753-64.

150. Wipke BT, Allen PM. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol. 2001;167(3):1601-8.

151. Solomon S, Rajasekaran N, Jeisy-Walder E, Snapper SB, Illges H. A crucial role for macrophages in the pathology of K/B x N serum-induced arthritis. Eur J Immunol. 2005;35(10):3064-73.

152. Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, Benoist C, Gravallese EM. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol. 2001;159(5):1689-99.

153. Buttgereit F, Zhou H, Kalak R, Gaber T, Spies CM, Huscher D, Straub RH, Modzelewski J, Dunstan CR, Seibel MJ. Transgenic disruption of glucocorticoid signaling in mature osteoblasts and osteocytes attenuates K/BxN mouse serum-induced arthritis in vivo. Arthritis and rheumatism. 2009;60(7):1998-2007.

154. Kleyer A, Finzel S, Rech J, Manger B, Krieter M, Faustini F, Araujo E, Hueber AJ, Harre U, Engelke K, Schett G. Bone loss before the clinical onset of rheumatoid arthritis in subjects with anticitrullinated protein antibodies. Annals of the Rheumatic Diseases. 2014;73(5):854.

155. van den Berg WB, Joosten LA, van Lent PL. Murine antigen-induced arthritis. Methods Mol Med. 2007;136:243-53.

156. Lens JW, van den Berg WB, van de Putte LB. Flare-up of antigen-induced arthritis in mice after challenge with intravenous antigen: studies on the characteristics of and mechanisms involved in the reaction. Clin Exp Immunol. 1984;55(2):287-94.

157. Bendele A, McAbee T, Sennello G, Frazier J, Chlipala E, McCabe D. Efficacy of sustained blood levels of interleukin-1 receptor antagonist in animal models of arthritis: Comparison of efficacy in animal models with human clinical data. Arthritis & Rheumatism. 1999;42(3):498-506.

158. Lee H, Zahra D, Vogelzang A, Newton R, Thatcher J, Quan A, So T, Zwirner J, Koentgen F, Padkjaer SB, Mackay F, Whitfeld PL, Mackay CR. Human C5aR knock-in mice facilitate the production and assessment of anti-inflammatory monoclonal antibodies. Nat Biotechnol. 2006;24(10):1279-84.

159. Seifer DR, Furman BD, Guilak F, Olson SA, Brooks SC, 3rd, Kraus VB. Novel synovial fluid recovery method allows for quantification of a marker of arthritis in mice. Osteoarthritis and cartilage. 2008;16(12):1532-8.

160. Spies CM, Wiebe E, Tu J, Li A, Gaber T, Huscher D, Seibel MJ, Zhou H, Buttgereit F. Acute murine antigen-induced arthritis is not affected by disruption of osteoblastic glucocorticoid signalling. BMC Musculoskelet Disord. 2014;15(1):31.

161. Yang YH, Morand EF, Getting SJ, Paul-Clark M, Liu DL, Yona S, Hannon R, Buckingham JC, Perretti M, Flower RJ. Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. Arthritis and rheumatism. 2004;50(3):976-84.

162. Sher LB, Woitge HW, Adams DJ, Gronowicz GA, Krozowski Z, Harrison JR, Kream BE. Transgenic Expression of 11β-Hydroxysteroid Dehydrogenase Type 2 in Osteoblasts Reveals an Anabolic Role for Endogenous Glucocorticoids in Bone. Endocrinology. 2004;145(2):922-9.

163. Tu J, Zhang Y, Kim S, Wiebe E, Spies CM, Buttgereit F, Cooper MS, Seibel MJ, Zhou H. Transgenic Disruption of Glucocorticoid Signaling in Osteoblasts Attenuates Joint Inflammation in Collagen Antibody-Induced Arthritis. Am J Pathol. 2016;186(5):1293-301.

164. Leech M, Metz C, Bucala R, MorAnd EF. Regulation of macrophage migration inhibitory factor by endogenous glucocorticoids in rat adjuvant-induced arthritis. Arthritis & Rheumatism. 2000;43(4):827-33.

165. Ignatius A, Schoengraf P, Kreja L, Liedert A, Recknagel S, Kandert S, Brenner RE, Schneider M, Lambris JD, Huber-Lang M. Complement C3a and C5a modulate osteoclast formation and inflammatory response of osteoblasts in synergism with IL-1β. Journal of Cellular Biochemistry. 2011;112(9):2594-605.

166. Coulpier M, Andreev S, Lemercier C, Dauchel H, Lees O, Fontaine M, Ripoche J. Activation of the endothelium by IL-1α and glucocorticoids results in major increase of complement C3 and factor B production and generation of C3a. Clinical & Experimental Immunology. 1995;101(1):142-9.

167. Cook AD, De Nardo CM, Braine EL, Turner AL, Vlahos R, Way KJ, Beckman SK, Lenzo JC, Hamilton JA. Urokinase-type plasminogen activator and arthritis progression: role in systemic disease with immune complex involvement. Arthritis Res Ther. 2010;12(2):R37.

168. Werb Z, Mainardi CL, Vater CA, Harris ED, Jr. Endogenous activation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. N Engl J Med. 1977;296(18):1017-23.

169. Murphy G, Atkinson S, Ward R, Gavrilovic J, Reynolds JJ. The role of plasminogen activators in the regulation of connective tissue metalloproteinases. Ann N Y Acad Sci. 1992;667(1):1-12.

170. Hecht M, Heider U, Kaiser M, von Metzler I, Sterz J, Sezer O. Osteoblasts promote migration and invasion of myeloma cells through upregulation of matrix metalloproteinases, urokinase plasminogen activator, hepatocyte growth factor and activation of p38 MAPK. Br J Haematol. 2007;138(4):446-58.

171. Yang YH, Carmeliet P, Hamilton JA. Tissue-type plasminogen activator deficiency exacerbates arthritis. J Immunol. 2001;167(2):1047-52.

172. Busso N, Peclat V, Van Ness K, Kolodziesczyk E, Degen J, Bugge T, So A. Exacerbation of antigen-induced arthritis in urokinase-deficient mice. J Clin Invest. 1998;102(1):41-50.

173. Santos LL, Fan H, Hall P, Ngo D, Mackay CR, Fingerle-Rowson G, Bucala R, Hickey MJ, Morand EF. Macrophage migration inhibitory factor regulates neutrophil chemotactic responses in inflammatory arthritis in mice. Arthritis and rheumatism. 2011;63(4):960-70.

174. Calandra T, Bernhagen J, Metz CN, Spiegel LA, Bacher M, Donnelly T, Cerami A, Bucala R. MIF as a glucocorticoid-induced modulator of cytokine production. Nature. 1995;377(6544):68-71.

175. Onodera S, Suzuki K, Matsuno T, Kaneda K, Kuriyama T, Nishihira J. Identification of macrophage migration inhibitory factor in murine neonatal calvariae and osteoblasts. Immunology. 1996;89(3):430-5.

176. Swolin-Eide D, Ohlsson C. Effects of cortisol on the expression of interleukin-6 and interleukin-1 beta in human osteoblast-like cells. J Endocrinol. 1998;156(1):107-14.

177. Buttgereit F. Views on glucocorticoid therapy in rheumatology: the age of convergence. Nature Reviews Rheumatology. 2020;16(4):239-46.

178. Van Staa TP, Leufkens HGM, Abenhaim L, Zhang B, Cooper C. Use of Oral Corticosteroids and Risk of Fractures. Journal of Bone and Mineral Research. 2000;15(6):993-1000.

179. Szekanecz Z, Halloran MM, Volin MV, Woods JM, Strieter RM, Haines III GK, Kunkel SL, Burdick MD, Koch AE. Temporal expression of inflammatory cytokines and chemokines in rat adjuvant-induced arthritis. Arthritis & Rheumatism. 2000;43(6):1266-77.

180. Miyazaki Y, Yokozeki H, Awad S, Igawa K, Minatohara K, Satoh T, Katayama I, Nishioka K. Glucocorticoids augment the chemically induced production and gene expression of interleukin-1alpha through NF-kappaB and AP-1 activation in murine epidermal cells. J Invest Dermatol. 2000;115(4):746-52.

181. Horiuchi Y, Bae SJ, Katayama I. FK506 (tacrolimus) inhibition of intracellular production and enhancement of interleukin 1alpha through glucocorticoid application to chemically treated human keratinocytes. Skin Pharmacol Physiol. 2005;18(5):241-6.

182. Elenkov IJ, Papanicolaou DA, Wilder RL, Chrousos GP. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. Proc Assoc Am Physicians. 1996;108(5):374-81.

183. Josephson MB, Jiao J, Xu S, Hu A, Paranjape C, Grunstein JS, Grumbach Y, Nino G, Kreiger PA, McDonough J, Grunstein MM. IL-13-induced changes in endogenous glucocorticoid metabolism in the lung regulate the proasthmatic response. Am J Physiol Lung Cell Mol Physiol. 2012;303(5):L382-90.

184. Imhof AK, Gluck L, Gajda M, Brauer R, Schaible HG, Schulz S. Potent anti-inflammatory and antinociceptive activity of the endothelin receptor antagonist bosentan in monoarthritic mice. Arthritis Res Ther. 2011;13(3):R97.

185. Monach P, Hattori K, Huang H, Hyatt E, Morse J, Nguyen L, Ortiz-Lopez A, Wu H-J, Mathis D, Benoist C. The K/BxN Mouse Model of Inflammatory Arthritis. In: Cope AP, editor. Arthritis Research: Methods and Protocols Volume 2. Totowa, NJ: Humana Press; 2007. p. 269-82.

186. Monach PA, Mathis D, Benoist C. The K/BxN Arthritis Model. Current Protocols in Immunology. 2008;81(1):15.22.1-15.22.12.

187. Akilesh S, Petkova S, Sproule TJ, Shaffer DJ, Christianson GJ, Roopenian D. The MHC class I–like Fc receptor promotes humorally mediated autoimmune disease. The Journal of Clinical Investigation. 2004;113(9):1328-33.

188. Sharp JT, Van Der Heijde D, Boers M, Boonen A, Bruynesteyn K, Emery P, Genant HK, Herborn G, Jurik A, Lassere M, McQueen F, Ostergaard M, Peterfy C, Rau R, Strand V, Wassenberg S, Weissman B, Subcommittee on Healing of Erosions of the OIC. Repair of erosions in rheumatoid arthritis does occur. Results from 2 studies by the OMERACT Subcommittee on Healing of Erosions. J Rheumatol. 2003;30(5):1102-7.

189. Rau R, Wassenberg S, Herborn G, Perschel WT, Freitag G. Identification of radiologic healing phenomena in patients with rheumatoid arthritis. J Rheumatol. 2001;28(12):2608-15.

190. Sokka T, Hannonen P. Healing of erosions in rheumatoid arthritis. Ann Rheum Dis. 2000;59(8):647-9.

191. Ideguchi H, Ohno S, Hattori H, Senuma A, Ishigatsubo Y. Bone erosions in rheumatoid arthritis can be repaired through reduction in disease activity with conventional disease-modifying antirheumatic drugs. Arthritis Res Ther. 2006;8(3):R76.

192. Walsh NC, Gravallese EM. Bone remodeling in rheumatic disease: a question of balance. Immunol Rev. 2010;233(1):301-12.

193. Walsh NC, Reinwald S, Manning CA, Condon KW, Iwata K, Burr DB, Gravallese EM. Osteoblast function is compromised at sites of focal bone erosion in inflammatory arthritis. J Bone Miner Res. 2009;24(9):1572-85.

6 APPENDIX

6.1 Statutory Declaration

"I, Edgar Wiebe, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "The role of endogenous glucocorticoid signaling in osteoblasts on inflammation and bone destruction by autoimmune arthritis", independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal

Code) are known to me.”

Date Signature

6.2 Declaration of own contribution to publications

Edgar Wiebe contributed the following to the below listed publications:

C. M. Spies, E. Wiebe, J. Tu, A. Li, T. Gaber, D. Huscher, M. J. Seibel, H. Zhou, F. Buttgereit. Acute murine antigen-induced arthritis is not affected by disruption of osteoblastic glucocorticoid signaling. BMC Musculoskelet Disord 2014 Feb 3;15:31. PMID: 24491163 [PubMed - indexed for MEDLINE].

Edgar Wiebe was involved in planning and conducting all experiments in the above-mentioned publication: He performed clinical assessment of arthritis, histological scoring and micro-CT as well as histomorphometry analysis. For all of these, he collected, prepared and interpreted the data. In addition, he was responsible for statistical analysis of histological, micro-CT and histomorphometry data. Based on these results, he created Figures 1-4 as depicted in the publication. He also gathered blood samples for serum cytokine analysis and performed statistical evaluation of the measurements as shown in Table 1. Thus, Edgar Wiebe was responsible for all results presented in the publication. He was also involved in writing and revising the manuscript.

_____

Signature, date and stamp of first supervising university professor / lecturer

_____

Signature of doctoral candidate

6.3 Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

6.4 List of publications

2020 Wiebe E, Freier D, Huscher D, Dallagiacoma G, Hermann S, Biesen R, Burmester G, Buttgereit F. Prevalence of Osteoporosis and Fragitlity Fractures Is Not Different Between ACPA Positive Patients Compared to ACPA Negative Patients in a Real World Setting, Despite Longer Disease Duration and Glucocorticoid-Treatment [abstract]. Arthritis Rheumatol. 2020; 72 (suppl 10).

2020 Wiebe E, Freier D, Huscher D, Hermann S, Biesen R, Buttgereit F. Glucocorticoid-Induced Osteoporosis in Patients with Chronic Inflammatory Rheumatic Diseases: A Multivariate Linear Regression Analysis Identifying Factors Affecting Bone Mineral Density [abstract]. Arthritis Rheumatol. 2020; 72 (suppl 10).

2018 E. Wiebe, R. Biesen, K.N. Zeiner, D.C. Freier, S. Hermann, G.-R. Burmester, F. Buttgereit.
Quantifying the treatment with glucocorticoids as a risk factor for the occurrence of osteoporosis and fractures in patients with RA.
Ann Rheum Dis 2018; 77(Suppl A):444

2017 E. Wiebe, C. Spies, J. Tu, T. Maleitzke, Y. Zhang, M. Seibel, H. Zhou, F. Buttgereit.
Transgenic disruption of glucocorticoid-signaling in mature osteoblasts and osteocytes attenuates structural bone damage in a long-term murine K/BxN serum-induced arthritis model.
Ann Rheum Dis 2017;76(Suppl 2):129.

2016 Tu J, Zhang Y, Kim S, Wiebe E, Spies CM, Buttgereit F, Cooper MS, Seibel MJ, Zhou H.
Transgenic Disruption of Glucocorticoid Signaling in Osteoblasts Attenuates Joint Inflammation in Collagen Antibody-Induced Arthritis.

Am J Pathol. 2016 May;186(5):1293-301. doi: 10.1016/j.ajpath.2015.12.025. Epub 2016 Mar 14.
PMID: 26988651 [PubMed - indexed for MEDLINE].

2014 C. M. Spies, E. Wiebe, J. Tu, A. Li, T. Gaber, D. Huscher, M. J. Seibel, H. Zhou, F. Buttgereit.
Acute murine antigen-induced arthritis is not affected by disruption of osteoblastic glucocorticoid signaling.
BMC Musculoskelet Disord 2014 Feb 3;15:31.
PMID: 24491163 [PubMed - indexed for MEDLINE].

Not related to this project:

2014 Alten R, Wiebe E.
Hypothalamic-pituitary-adrenal axis function in patients with rheumatoid arthritis treated with different glucocorticoid approaches.
Neuroimmunomodulation. 2015;22(1-2):83-8. doi: 10.1159/000362731. Epub 2014 Sep 12. Review.
PMID: 25228310 [PubMed - indexed for MEDLINE].

6.5 Acknowledgements

I would like to thank Professor Frank Buttgereit for having chosen me to take part in the cooperative research program with the ANZAC Research Institute in Sydney and having granted me a fellowship for this purpose and even beyond the research stay. This has shaped my way into the realm of osteoimmunological research which I continue to pursue today under his guidance. My thanks extend to Professor Hong Zhou and Professor Markus Seibel of the Bone Biology Program at the ANZAC Research Institute for creating a fruitful environment with numerous critical discussions and encouraging ideas.

I am grateful to the entire Bone Biology team for being supportive, curious and amiable. It has always been a great pleasure working together, exchanging ideas and learning from each other. I would like to highlight the help by Dr. Aiqing Li who conducted preliminary studies of the AIA model and instructed us in the applied methods, as well as the help from Julian Kelly with histomorphometry and micro-CT. Also, I thank Manuela Jakstadt for excellent technical assistance in cytokine analysis, Dr. Dörte Huscher for her very important statistical expertise and especially Dr. Timo Gaber for supporting the continuation of the work at the Rheumatology labs at the Charité with a lot of logistic help and intellectual curiosity. My special thanks go to Dr. Jinwen Tu, who was always a reliable and assiduous companion in the conduction of experiments.

I am deeply indebted to Dr. Cornelia Spies with whom I have conducted most experiments and analyses together. She has accompanied me throughout the research stay and beyond, being a great teacher, mentor and role model. I am grateful for her guidance and look back with a smile at the many days of the year we worked in sterile quarantine conditions of the animal house, having meaningful conversations beyond science and medicine.

Last but not least, I am endlessly thankful to my parents David and Larissa Wiebe for supporting me throughout life, shaping me in who I am and what I strive for, and for creating a basis for achieving my goals. It is to them, that I dedicate this work.

And finally, I am thankful to my wife, Anne Carina, for having accompanied me from the beginning of the research stay until now, and our three kids, Elena, Jakob and Lara, for having been the best imaginable excuse for keeping me from finishing this work.