Tampere University

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Macrophage and Chondrocyte Phenotypes in Inflammation

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ACADEMIC DISSERTATION
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The Immunopharmacology Research Group, Faculty of Medicine and Health Technology, Tampere University and Tampere University Hospital
Finland

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CONTENTS

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AUTHOR'S CONTRIBUTION

The author contributed to the original communications in following ways:

In **Study I**, the author participated in the conceptualization and design of the study, performed a part of the laboratory experiments, analyzed the data and drafted the manuscript.

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ABBREVIATIONS

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AMPK	AMP activated protein kinase
AP-1	activator protein 1
ArgI	arginase 1
BMP	bone morphogenetic protein
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
C/EBP	CCAAT-enhancer-binding protein
C(IFNγ)	interferon gamma -induced chondrocyte phenotype
C(IL-1β)	interleukin 1 beta -induced chondrocyte phenotype
C(IL-4)	interleukin 4 -induced chondrocyte phenotype
C(IL-17)	interleukin 17 -induced chondrocyte phenotype
COX	cyclo-oxygenase
CREB	cAMP response element-binding protein
CSF	colony stimulating factor
C_T	cycle threshold
DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
DUSP	dual specificity phosphatase
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal regulated kinase
FC	fold change
FDR	false discovery rate
FGF	fibroblast growth factor
Fizz1	found in inflammatory zone 1
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GDF	growth differentiation factor
GO	Gene Ontology
GR	glucocorticoid receptor
GWAS	genome wide association study
GWEA	genome wide expression analysis
HE	hematoxylin-eosin
HIF	hypoxia-inducible factor
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin

IGF	insulin-like growth factor
IL	interleukin
iNOS	inducible nitric oxide synthase
IPA	Ingenuity Pathway Analysis
IRF	interferon regulatory factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KLF	Krüppel-like factor
KO	knock-out
LPS	lipopolysaccharide
LXR	liver X receptor
M1	classically activated macrophage
M2	alternatively activated macrophage
MAPK	mitogen-activated protein kinase
MKK	mitogen-activated protein kinase kinase
MKKK	mitogen-activated protein kinase kinase kinase
miRNA	microRNA
MKP-1	mitogen-activated protein kinase phosphatase 1
MMP	matrix metalloproteinase
mPGES-1	microsomal prostaglandin E synthase-1
MRC1	mannose receptor 1
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NGS	next generation sequencing
NK	natural killer (T cell)
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	nonsteroidal anti-inflammatory drug
OA	osteoarthritis
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PG	prostaglandin
PI3K	phosphoinositide 3-kinase
PM	peritoneal macrophage
pSTAT	phosphorylated signal transducer and activator of transcription
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species

RT-qPCR	quantitative reverse transcription polymerase chain reaction
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SHIP	phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase
SSc	systemic sclerosis
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
T_H cell	T helper lymphocyte
T_H1 cell	Type 1 T helper lymphocyte
T_H2 cell	Type 2 T helper lymphocyte
T_H17 cell	Type 17 T helper lymphocyte
TIMP	tissue inhibitor of metalloproteinases
TLR	toll-like receptor
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WB	western blot
WT	wild-type

In accordance with standard biochemical nomenclature, gene names are written in *cursive* unless indicated otherwise. The protein products of these genes are written in print. Likewise, in accordance with standard nomenclature, human gene names are written in *ALL CAPS* and murine gene names in *Sentence case*.

ABSTRACT

In the human body, several cell types can adopt different phenotypes in response to their environment. One of the most well-established examples of this heterogeneity is the $T_H1/T_H2/T_H17$ paradigm of T helper lymphocytes. An analogous paradigm is the macrophage polarization, which has the proinflammatory "classically activated" (M1) macrophages on the one end of the continuum and the anti-inflammatory "alternatively activated" (M2) cells on the other. Several different factors, such as endogenous cytokines, intracellular signaling mechanisms, metabolic factors and medications, may modulate macrophage phenotype.

Osteoarthritis (OA) is a common and hard-to-treat disease characterized by the presence of chronic low-grade inflammation in the affected joint. Chondrocytes, the cells of articular cartilage, are known to undergo changes in their gene expression in OA. However, these changes, as well as their response to endogenous factors and exogenous therapeutics, are only partially known. In this thesis, it was hypothesized that chondrocytes adopt distinct phenotypes induced by different endogenous and exogenous factors.

Part I of this study concerns the effects of mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1), which has previously been shown to attenuate inflammation, on M2 macrophage activation. In macrophages from MKP-1 deficient mice, the expression of M2 markers was reduced compared to cells from wild-type animals. Furthermore, the effects of the glucocorticoid dexamethasone on both M1 and M2 markers were attenuated in macrophages from MKP-1 deficient mice. These results show that instead of merely attenuating M1 activation, MKP-1 actually "shifts" macrophage phenotype towards M2, and that the effects of glucocorticoids on macrophage phenotype are mediated, at least partly, via MKP-1.

In part II, effects of nortrachelogenin, a major lignan component of *Pinus sylvestris* knot extract, on macrophage phenotype were studied. Nortrachelogenin was found to attenuate alternative (M2) activation and to reduce bleomycin-induced dermal fibrosis in mice, indicating therapeutic potential in fibrosing diseases.

In part III, chondrocyte phenotypes induced by the central $T_H1/T_H2/T_H17$ cytokines (IFNγ, IL-1, IL-4 and IL-17) were introduced by treating chondrocytes isolated from OA patients with these cytokines. Cells treated with IL-1β [C(IL-1β) chondrocyte phenotype] were characterized by the expression of a large number of proinflammatory and catabolic genes, while a smaller set of proinflammatory genes was induced in the C(IL-17) chondrocyte phenotype. The genes upregulated in the C(IFNγ) chondrocyte phenotype were relatively distinct from those induced by the two other proinflammatory cytokines and included those associated with antigen processing and presentation. In the C(IL-4) chondrocyte phenotype, a modest number of genes was differentially expressed compared with control chondrocytes. These genes were mostly those linked to regulation of inflammation, transforming growth factor beta (TGFβ) signaling and cell proliferation.

Part IV of the study describes the effects of glucocorticoids on chondrocyte phenotype. In OA chondrocytes, the glucocorticoid dexamethasone significantly and markedly (with fold change > 2.0) affected the expression of over 1200 genes associated with, among others, inflammation, cartilage anabolism and catabolism, as well as lipid and carbohydrate metabolism. When the results were compared with previous genome-wide expression analyses (GWEAs) studying chondrocyte transcriptome in degraded and preserved OA cartilage, dexamethasone was shown to partly shift chondrocyte gene expression profile from degraded towards preserved cartilage. As intra-articular glucocorticoid injections are widely used in the treatment of OA, these results show that glucocorticoids have wide-ranging effects on chondrocyte phenotype and partly normalize chondrocyte gene expression towards healthy cartilage. The results also inform future studies on the effects of glucocorticoids on cartilage health.

In part V, the effects of the widely used nonsteroidal anti-inflammatory drug ibuprofen on OA chondrocyte phenotype were studied. Ibuprofen alone had no significant effects on gene expression, but when given together with the proinflammatory cytokine IL-1β, a markedly differential expression (fold change > 1.5 in either direction) of 93 genes was observed. The upregulated genes included anti-inflammatory factors (such as *PPARG* and *IL10RA*) and genes associated with cell adhesion, while inflammatory factors such as *IL23A* and *IL6* were among the downregulated genes.

The results demonstrate the ability of both macrophages and chondrocytes to adopt various phenotypes in inflammatory conditions and alter them in response to diverse medications and environmental factors. This study constitutes the first characterization of "polarized" chondrocyte phenotypes induced by the central $T_H1/T_H2/T_H17$ cytokines. Also, the (OA) chondrocyte phenotypes induced by two widely used classes of OA medications, glucocorticoids and nonsteroidal anti-inflammatory drugs, were comprehensively characterized for the first time. The results enhance our understanding of the role of macrophages and chondrocytes in inflammation and as targets of drug development. They also provide a starting point for more detailed hypothesis-directed studies in the future.

TIIVISTELMÄ

Ihmiskehon useat solutyypit voivat omaksua erilaisia ilmiasuja (fenotyyppejä) reaktiona ympäristön signaaleihin. Eräs parhaiten tunnetuista tätä ilmiötä kuvaavista esimerkeistä on ns. auttaja-T-lymfosyyttien (T_H-solujen) $T_H1/T_H2/T_H17$-ilmiasujako. Vastaavanlainen jako, makrofagipolarisaatio, on kehitetty kuvaamaan makrofagien ilmiasuja. ”Klassisesti aktivoituneet” eli M1-makrofagit edistävät tulehdusta, ”vaihtoehtoisesti aktivoituneet” M2-makrofagit puolestaan hillitsevät sitä. Useiden eri tekijöiden, kuten sytokiinien, solunsisäisten signalointireittien, metabolisten tekijöiden ja lääkkeiden, on osoitettu vaikuttavan makrofagien ilmiasuun.

Nivelrikko on yleinen ja vaikeahoitoinen tauti, jolle on tyypillistä nivelen krooninen, matala-asteinen tulehdustila. Geenien ilmentymisen nivelruston soluissa, kondrosyyteissä, on havaittu muuttuvan tautiprosessin edetessä. Nämä muutokset, ja niiden vasteet erilaisiin elimistön sisäisiin tekijöihin ja lääkityksiin, tunnetaan kuitenkin puutteellisesti. Tässä väitöskirjassa esitettiin hypoteesi, jonka mukaan rustosolut omaksuvat erilaisia ilmiasuja erilaisten tulehdustekijöiden ja lääkkeiden vaikutuksesta.

Tämän väitöskirjan osatyössä I tutkimme tunnetun tulehdusreaktiota hillitsevän tekijän, MAP-kinaasifosfataasi 1:n (MKP-1:n), vaikutuksia M2-makrofagiaktivaatioon. MKP-1-puutteisilla hiirillä useiden M2-makrofageille tyypillisten geenien ilmentyminen oli villityypin hiiriä vähäisempää. Lisäksi glukokortikoidi deksametasonin vaikutukset sekä tyypillisiin M1- että M2-geeneihin olivat MKP-1-puutteisten hiirten makrofageissa villityyppiä vähäisempiä. Nämä tulokset osoittavat, että MKP-1 ei ainoastaan hillitse M1-aktivaatiota, vaan ”siirtää” makrofagiaktivaatiota tulehdusta rauhoittavaan M2-suuntaan, ja että glukokortikoidien vaikutukset makrofageissa välittyvät ainakin osittain MKP-1:n kautta.

Osatyössä II tutkimme nortrakelogeniinin, männyn (*Pinus sylvestris*) sisäoksauutteen merkittävän lignaanikomponentin, vaikutuksia makrofagien ilmiasuun. Yhdiste

hillitsi M2-aktivaatiota ja vaimensi ihofibroosin kehittymistä skleroderman kokeellisessa mallissa. Tämä viittaa siihen, että nortrakelogeniini voisi olla hyödyllinen yhdiste fibrotisoivien sairauksien hoidossa.

Osatyössä III nivelrikkopotilailta eristettyjä rustosoluja käsiteltiin keskeisillä $T_H1/T_H2/T_H17$-sytokiineillä, ja tavoitteena oli tutkia näiden sytokiinien vaikutuksia rustosolujen ilmiasuun. IL-1β:llä käsitellyissä soluissa [C(IL-1β)-rustosoluissa] useiden tulehdusta edistävien ja katabolisten geenien ilmentyminen oli lisääntynyt. C(IL-17)-ilmiasussa oli indusoitunut rajatumpi joukko tulehdusta edistäviä geenejä. C(IFNγ)-ilmiasu poikkesi varsin paljon kahdesta edellisestä; useiden antigeenien käsittelyyn ja esittelyyn liittyvien geenien ilmentyminen oli siinä lisääntynyt. C(IL-4)-ilmiasussa varsin pienen geenijoukon ilmentyminen erosi kontrollisoluista. Erityisesti tulehdusta hillitsevien, TGFβ-signalointiin osallistuvien ja solunjakautumiseen liittyvien geenien ilmentymisessä havaittiin muutoksia C(IL-4)-rustosoluissa kontrollisoluihin verrattuna.

Osassa IV tutkittiin glukokortikoidien vaikutuksia rustosolujen ilmiasuun. Nivelensisäisiä glukokortikoidiruiskeita käytetään laajalti nivelrikon pahenemisvaiheiden hoidossa, mutta niiden pitkäaikaisvaikutukset rustoon ja taudin etenemiseen ovat pitkälti tuntemattomia. Nivelrikkorustosta eristetyissä rustosoluissa deksametasoni vaikutti merkittävästi suuren geenijoukon ilmentymiseen; yli 1200 geenissä havaittiin yli kaksinkertainen, tilastollisesti merkitsevä ilmentymisero deksametasonille altistettujen ja kontrollisolujen välillä. Näihin kuului mm. tulehdusreaktioon, rustomatriksin synteesiin ja hajoamiseen sekä lipidi- ja hiilihydraattimetaboliaan liittyviä geenejä. Kun tuloksia verrattiin aiempiin genominlaajuisiin ilmentymisanalyysitutkimuksiin, joissa oli tutkittu geenien ilmentymisen eroja vaurioituneen ja makroskooppisesti normaalin nivelrikkoruston välillä, deksametasonin havaittiin osittain muokkaavan rustosolujen ilmiasua vaurioituneesta rustosta normaalimman ruston suuntaan. Tulokset osoittavat, että glukokortikoideilla on laaja-alaisia vaikutuksia nivelrikkopotilaan rustosolujen ilmiasuun, ja että ne osin normalisoivat rustosolujen geenieksprессiota vaurioituneesta rustosta normaalimman ruston suuntaan.

Osassa V tutkimme laajasti käytetyn tulehduskipulääke ibuprofeenin vaikutuksia rustosolujen ilmiasuun. Ibuprofeenilla ei ollut yksin merkitsevää vaikutusta geenien ilmentymiseen, mutta kun soluja stimuloitiin tulehdusta edistävällä sytokiini IL-1β:lla, ibuprofeeni vaikutti selvästi (aiheutti yli 1.5-kertaisen muutoksen) 93 geenin

ilmentymiseen. Useiden tulehdusta hillitsevien (kuten *PPARG*:n and *IL10RA*:n) ja soluadheesioon liittyvien geenien ilmentyminen lisääntyi, kun taas useiden tulehdustekijöiden (kuten *IL23A*:n and *IL6*:n) ilmentyminen väheni.

Väitöstutkimuksen tulokset osoittavat, että sekä makrofagit että rustosolut ilmentävät erilaisia ilmiasuja tulehdusreaktiossa. Ilmiasut voivat myös muuttua lääkkeiden ja ympäristötekijöiden vaikutuksesta. Tässä väitöstutkimuksessa esitellään ensimmäistä kertaa keskeisten $T_H1/T_H2/T_H17$-sytokiinien indusoimat rustosolujen ilmiasut. Nivelrikossa laajalti käytettyjen lääkkeiden, glukokortikoidien ja tulehduskipulääkkeiden, vaikutuksia nivelrikkopotilailta eristettyihin rustosoluihin tutkittiin, ja näiden lääkkeiden indusoimat rustosolujen ilmiasut kuvattiin ensimmäistä kertaa. Tulokset lisäävät tietämystämme makrofagien ja rustosolujen merkityksestä tulehduksessa ja lääkevaikutuskohteina. Ne myös tarjoavat lähtöpisteen tarkemmille, hypoteesiohjatuille tutkimuksille.

1 INTRODUCTION

Inflammation is a process aiming to protect the body from external threats: microbes, parasites and other harmful insults. Owing to the large number and diversity of those threats, the human body has evolved a wide variety of cells that participate in inflammatory reactions. Many of these cells can also assume different phenotypes and gene expression profiles in order to effectively perform different tasks. An example is the concept of macrophage polarization, in which proinflammatory "M1" macrophages fight invading microbes, while the anti-inflammatory "M2" macrophages promote resolution of inflammation and tissue healing. Despite its vital defensive function, inflammation also has a dark side: inappropriately targeted or dysregulated inflammation can lead to development of illnesses such as autoimmune or allergic diseases. Another example of a disease associated with chronic inflammation is osteoarthritis (OA), a process characterized by constant low-grade inflammation in synovial joints, episodic inflammatory exacerbations, and degradation of articular cartilage.

A synovial joint is composed of a synovial capsule (itself consisting of a fibrous outer capsule and inner synovial membrane) as well as hyaline cartilage that covers articulating surfaces of the bones (Figure 1-1). Chondrocytes are the cells of cartilage, while the synovium contains macrophage-like type A and fibroblast-like type B synoviocytes, and bone is maintained by the actions of osteoblasts and osteoclasts (Scanzello & Goldring, 2021). All of these cells and tissues are involved in OA development. Cartilage covering bone surfaces is degraded and thinned, the subchondral bone undergoes sclerosis and may develop bony outgrowths (osteophytes), and the synovium displays variable degrees of inflammation.

At the cellular level, OA involves alterations in the phenotypes of different cells in the joint. Synovial cells of both A and B types assume proinflammatory phenotypes (Chou et al. 2020), and chondrocyte gene expression undergoes several changes with alterations in both ana- and catabolic functions. As OA progresses, chondrocytes are also increasingly lost to apoptosis. Increased amounts of several proinflammatory cytokines [such as interleukins (IL) 1β and 17] and catabolic factors [such as matrix

metalloproteinases (MMPs) 1 and 13] can be detected in the synovial fluid of OA patients, and increased serum levels of proinflammatory cytokines and adipokines link OA to systemic inflammatory states such as obesity. (Di Cesare et al., 2021)

OA causes widespread suffering and disability throughout the world, and despite intense search, no effective disease-modifying treatments are yet available. One potential therapeutic approach would be modulating the phenotypes of the cells in the joint. This approach is inspired by both physiology and pharmacology. T helper lymphocytes (T_H cells) can adopt both pro-inflammatory and anti-inflammatory phenotypes in response to different cytokines and environmental signals, and this has been extended to macrophages: the concept of macrophage polarization has proinflammatory and catabolic "classically activated" macrophages and the anti-inflammatory and healing-promoting "alternatively activated" macrophages as the two ends of the phenotypic spectrum (Hume, 2015). Also, several anti-inflammatory medications have been shown to work, at least partly, via modulating macrophage phenotype towards alternative activation (Ross et al., 2021).

Extending the phenotypic polarization of T_H cells and macrophages to chondrocytes is a novel approach for studying the pathophysiology of, and potential treatments for, OA. There are some hints that OA chondrocytes resemble classically activated macrophages, which raises an intriguing possibility: could they also assume an inflammation-quenching, potentially even reparative, phenotype? Moreover, could medications commonly used in OA have beneficial effects on chondrocyte phenotype? Modern methods such as RNA sequencing (RNA-Seq), which theoretically allows for a simultaneous quantification of every mRNA transcript in a sample, show particular promise for studying these issues.

This study investigates the phenotypes of macrophages and chondrocytes as well as the modulation of these phenotypes by different endo- and exogenous factors. Furthermore, the concept of macrophage polarization is extended to chondrocytes to provide a novel viewpoint on OA pathophysiology and potential treatments.

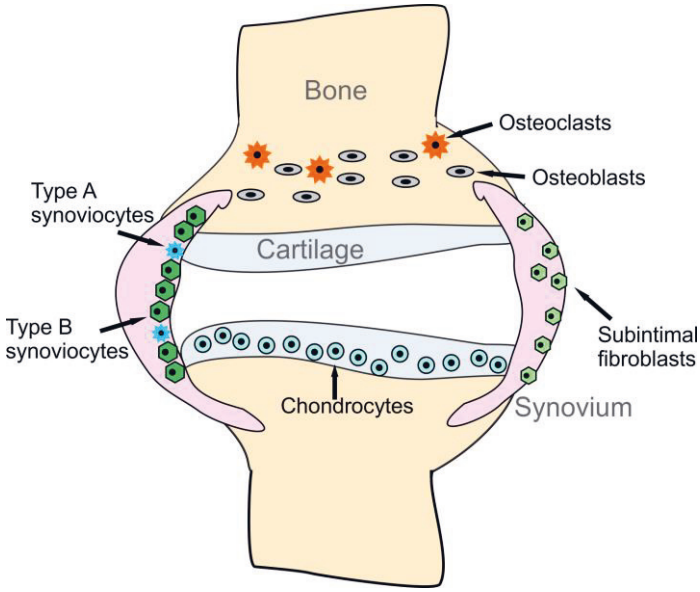

Figure 1-1. Schematic presentation of the structure of a healthy synovial joint. Bone homeostasis is maintained by osteoblasts and osteoclasts, while chondrocytes are the only resident cells in cartilage. The synovium contains a more diverse array of cells: the superficial layer (intima) is populated by macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes, while the deeper subintimal layer contains mainly subintimal fibroblasts.

2 REVIEW OF LITERATURE

2.1 Inflammation

Inflammation is a process aimed at defending the body against both internal and external threats. Closely linked with the concept of immunity, it involves the activation of immune system. Both innate and adaptive branches of the immune system play a central role in inflammation. *Innate immunity* consists of mechanical barriers, simple chemical defense mechanisms and phagocytic immune cells. Mechanicals barriers, such as skin and mucous membranes, directly prevent many pathogens and harmful agents from entering the body. Chemical defense systems include, among others, proteolytic enzymes present on mucous membranes, gastric acid and the complement cascade; factors that destroy pathogens or hinder their function. Phagocytic cells include macrophages, neutrophils and dendritic cells. These cells engulf microbes and other foreign particles, subsequently destroying them with reactive oxygen species (ROS), reactive nitrogen species (RNS) and proteolytic enzymes. (Abbas et al., 2018a; Kumar et al., 2021)

In contrast to the general nature of innate immunity, *adaptive immunity* is a system for combating specific pathogens and other harmful agents. Phagocytic cells "present" antigens (pieces of phagocytosed entities) to lymphocytes, activating them. This leads to production of proapoptotic factors by cytotoxic T lymphocytes and antibodies by B lymphocytes. After the resolution of inflammation, some of the activated lymphocytes persist in the body as memory cells, allowing the immune system to rapidly react to a potential reinfection by the same agent. True to its name, the system therefore adapts to fight off the threats it has encountered in the past. (Abbas et al., 2018a; Kumar et al., 2021)

Inflammatory reactions can also be classified by their time scale. *Acute inflammation* is a transient reaction towards an insult such as an invading pathogen. It is initiated by immune cells already present in the tissue such as tissue macrophages or dendritic cells. These cells possess pattern recognition receptors (PRRs), which are activated by compounds derived either from an invading pathogen (pathogen-associated

molecular patterns or PAMPs) or damaged host tissues (damage-associated molecular patterns or DAMPs). (Kumar et al., 2021). These cells produce large amounts of proinflammatory cytokines, attracting more immune cells to the site of inflammation. This is aided by distention and increased permeability ("leaking") of local blood vessels. Inflammatory cells migrating into tissues during the early phase of inflammation consist mainly of neutrophils. Circulating monocytes are also recruited into the tissue and differentiate into macrophages (mainly of the M1 phenotype, see p. 27). The later stages of inflammation are characterized by the arrival of lymphocytes and additional macrophages. In addition to cell-mediated responses, several acellular processes are also activated. These include complement, coagulation and fibrinolytic systems. (Abbas et al., 2018a; Kumat et al., 2021)

Once the initial insult has been rendered harmless, the *resolution* phase of inflammation begins. This process is mainly coordinated by macrophages displaying alternatively activated (M2) phenotype (see p. 31). Aforementioned changes in vascular diameter and permeability are reversed, and recruitment of new immune cells ends. Tissue repair processes are initiated and fibroblasts are activated; this is aimed at repairing damage caused by both initial insult and the subsequent immune response. (Abbas et al., 2018a; Headland & Norling, 2015)

Occasionally, the immune system is unable to expunge the offending factor, or resolving processes otherwise fail to completely terminate the inflammation. This leads to *chronic inflammation*. A classic tissue-scale manifestation of chronic inflammation is a granuloma, a nodular collection of macrophages and T cells gathered around an immunogenic structure that couldn't be removed during acute inflammation, "walling it off" from the rest of the body (Abbas et al., 2018a). Chronic inflammation is also observed in autoimmune disorders such as rheumatoid arthritis and thyroiditis. Osteoarthritis (OA) is likewise an example of a chronic inflammatory disease. During recent decades, a more subtle form of chronic inflammation has garnered considerable interest. This "silent" or "low-grade" inflammation is marked by moderately increased plasma concentrations of proinflammatory cytokines and systemic activation of immune cells, and it has been linked to multiple common disorders such as obesity, diabetes mellitus type 2, atherosclerosis and Alzheimer's disease. This form of chronic inflammation is not driven by immune cells activated by microbes, but rather by endogenously produced factors. For example, adipose tissue secretes a variety of proinflammatory substances called adipokines, which potentially explain (at least part of) the well-established link

between obesity and its comorbidities such as diabetes mellitus type 2 and osteoarthritis (Abbas et al., 2018a; Azamar-Llamas et al., 2017).

The various phases and facets of inflammation are directed by a variety of cell types and their phenotypes. Classically, T helper (T_H) cells are considered important examples of phenotypic diversity, as they can adopt distinct phenotypes in response to different phases of inflammation and tissue environments. Different T_H phenotypes are associated with different cytokines (soluble extracellular "messenger" proteins). The T_H1 phenotype promotes inflammation, cell-mediated immunity and intracellular pathogen clearance. These cells are induced by interleukin 12 (IL-12) (secreted by macrophages, dendritic cells, neutrophils and/or B lymphocytes) and produce mainly interferon gamma (IFNγ) and IL-2 as effector cytokines. They induce macrophages to produce IL-1β, which in turn promotes the proinflammatory effects of T_H1 cells (Dinarello, 2009). T_H2 cells are induced by IL-2 and IL-4. They promote humoral immunity, defense against extracellular pathogens (such as helminths) and regulation of inflammation, but are also associated with allergies. These effects are mainly exerted via the secretion of IL-4 and IL-13, which in turn drive healing-promoting alternative (M2-type) macrophage activation (see p. 31) (Abbas et al., 2018b). A third well-defined T_H phenotype is the T_H17 cell. These cells are characterized by their ability to produce the proinflammatory cytokine IL-17, and they are induced by transforming growth factor beta (TGFβ), IL-6, IL-21 and IL-23. In their physiological role, T_H17 cells contribute to pathogen clearance from mucosal surfaces. However, they are also closely linked to autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis and psoriasis (Singh et al., 2013). Relative abundances of different T_H phenotypes can be thought to determine the current "working mode" of immune system. Accordingly, biological agents that target cytokines associated with different T_H phenotypes (such as IL-1 receptor antagonist and IL-17 monoclonal antibodies) are used as effective treatments of inflammatory diseases (Shepard et al., 2017).

2.2 Macrophages

Macrophages are versatile, multifunctional cells with several different roles in physiology and inflammation. Macrophage-like cells are found throughout the body: circulating monocytes in the blood can migrate into tissues and differentiate into macrophages when needed, and tissues are already "seeded" with tissue

macrophages or macrophage-like cells such as dendritic cells in the connective tissues, microglia in the brain and osteoclasts in the bones. Together these cells form the mononuclear phagocyte system, formerly known as reticuloendothelial system. (Abbas et al. 2018c, Wynn et al., 2013).

In the normal state, macrophages use phagocytosis to clear tissues of apoptotic cells and debris, participate in recycling of nutrients (e.g. iron) and contribute to tissue remodeling. In the setting of infection, macrophages phagocytose microbes and present their antigens to T cells, acting as links between innate and adaptive immunity. They also coordinate and regulate the actions of other leukocytes and tissues by secreting cytokines and other signaling mediator. The exact nature of produced signals depends on the nature and phase of inflammatory response. (Abbas et al. 2018c, Ginhoux & Jung, 2014)

Monocytes are circulating leukocytes that are produced from hematopoietic stem cells in the bone marrow. These cells typically circulate in the bloodstream for one to three days, after which they migrate (extravasate) to tissues. In addition to those in the blood, a large population of monocytes is held in "reserve" in the spleen (Swirski et al., 2009). Tissue macrophages can also renew from local stem cells, originally derived from embryonic precursors and "seeded" into tissues during development (Ginhoux & Jung, 2014). Nowadays, most tissue macrophages are thought to be of local origin in the physiological state. Especially in the setting of infection and inflammation, however, these populations are bolstered by circulating monocytes which mature into macrophages in the tissue (Jenkins & Hume, 2014). Thus, both of these processes appear to play a central role in the maintenance of tissue macrophage populations (Zhao et al., 2018).

2.2.1 Macrophage phenotypes

Initially, macrophage function was thought to be limited to phagocytizing pathogens and stimulating the other parts of the immune system. However, during the recent decades, it has been increasingly recognized that macrophages performing different roles display distinct phenotypes and transcriptomes. An influential paradigm representing this divide is modeled after the well-established $T_H1/T_H2/T_H17$ paradigm for T_H lymphocytes, central regulators of adaptive immunity (Figure 2-1). M1 macrophages, analogous to T_H1 cells, promote inflammation and defend the body against intracellular bacteria and protozoa. M2 macrophages, in turn, are

thought to have evolved to combat helminths and extracellular parasites. They also promote resolution of inflammation and tissue healing, preventing excessive M1/T_H1 -type responses from causing excessive harm to the body (Male et al., 2021). Some authors have extended this paradigm to IL-17 stimulated M17 macrophages. This macrophage phenotype is less well established than the M1/M2 scheme; these cells have been reported to promote inflammation in the early parts of infection or inflammatory response, but later participate in the clearance of apoptotic cells and the resolution of inflammation. They may also promote T_H17 responses and be involved in autoimmunity (Raucci et al., 2022; Zizzo & Cohen, 2013).

The model of macrophage polarization presented above has attracted criticism during the last years. It has been noted that the nomenclature has become confused, and terms for the phenotypes (especially the "alternative" ones) are used somewhat inconsistently (Hume, 2015; Orecchioni et al., 2019). Also, a rough M1/M2/(M17) division doesn't seem to be supported by evidence obtained from powerful transcriptomic technologies (Hume, 2015). This problem is exacerbated by the differences in currently known (or postulated) alternative activation markers between human and murine cells (see p. 37). However, in spite of its shortcomings, the concept of macrophage polarization appears to capture at least part of the differences of macrophage phenotypes observed in different states (Male et al., 2021, Martinez & Gordon, 2014). Thus, it can nevertheless be helpful for understanding the actions of macrophages in physiology and disease.

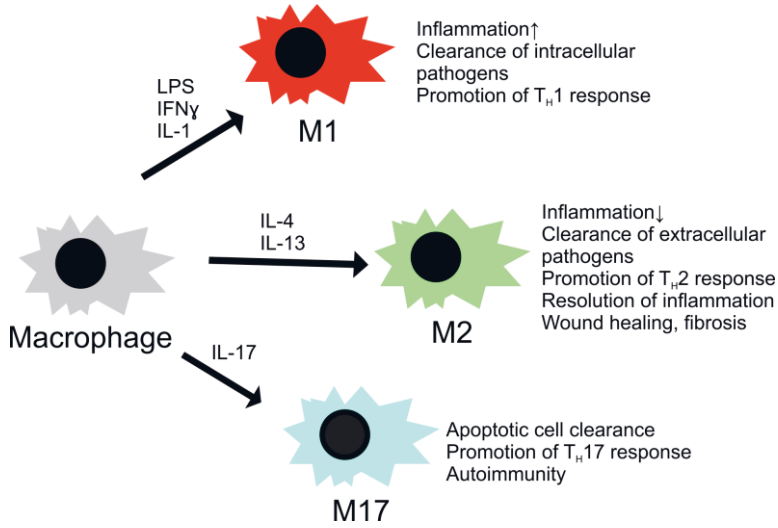

Figure 2-1. Macrophage phenotypes, their inducers and effects. The classical (M1) phenotype is induced by various endo- and exogenous proinflammatory stimuli; it further promotes inflammation and clearance of intracellular pathogens. The alternative (M2) phenotype is induced by the T_H2 cytokines IL-4 and IL-13; it promotes the resolution of inflammation, wound healing (which may proceed to fibrosis) and clearance of extracellular pathogens. The M17 phenotype is induced by the T_H17 cytokine IL-17, and it has been implicated to play a role in autoimmunity and the clearance of apoptotic cells.

2.2.2 Classical macrophage activation

Classically activated (M1) macrophages are activated by microbial products (such as lipopolysaccharide, LPS) and proinflammatory cytokines associated with T_H1 response (such as IFNγ, TNFα and IL-1). They effectively phagocytize microbes, destroy intracellular pathogens and produce proinflammatory and chemotactic mediators, thus recruiting other immune cells and promoting inflammation.

Mechanisms of classical activation

Of the various stimuli encountered by macrophages, one that appears to be most comprehensively characterized as an inducer of classical activation is IFNγ. Binding of this mediator to its receptor triggers Janus kinase (JAK) -mediated tyrosine phosphorylation and dimerization of the transcription factor signal transducer and activator of transcription 1 (STAT1) (Lawrence & Natoli, 2011). This factor then binds to cis-regulatory elements (so-called gamma-activated sequences) of several

proinflammatory genes such as *INOS*, class II major histocompatibility complex transactivator (*CIITA*) and *IL12* (Green et al., 2017).

A second major route to classical activation is toll-like receptor 4 (TLR4) signaling. This receptor belongs to the family of PRRs, utilized by the innate immunity system to detect constituents of invading microbes. The most well-known target for TLR4 is LPS, a component of cell walls of gram-negative bacteria (Vaure & Liu, 2014). It can also be activated by other microbial proteins as well as endogenous factors such as low-density lipoprotein and saturated fatty acids (Brubaker et al., 2015). Three other proteins facilitate the activation of TLR4 by LPS: lymphocyte antigen 96 (LY96, also known as MD2), CD14 and LPS binding protein (LBP) (Shimazu et al., 1999; Thomas et al., 2002). Upon LPS binding, conformational changes in TLR4 lead to activation of the Toll/IL-1 receptor (TIR) homology domain, an intracellular signaling domain shared by several parts of the TLR4-activated signaling cascade. Two major signaling pathways have been identified: the Myeloid Differentiation Primary Response Gene 88 (MyD88) -dependent pathway is regulated by the titular protein along with TIR domain-containing adaptor protein (TIRAP). They activate IL-1 receptor-associated kinases (IRAKs) as well as TNF receptor-associated factor 6 (TRAF6). The latter factor activates TAK1 (transforming growth factor-β-activated kinase 1), in turn leading to the activation of mitogen-activated protein kinases (MAPKs), central intracellular drivers of inflammation, and IκB Kinase (IKK). MAPKs then activate the transcription factor activator protein 1 (AP-1), while IKK induces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). These transcription factors then initiate the transcription of various proinflammatory effectors and other markers of classical activation. The other major TLR4 pathway is independent of MyD88, instead involving the adaptor proteins TIR-domain-containing adaptor inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). These act together to activate interferon regulatory factor 3 (IRF3), which then induces the production of type 1 interferons (Funami et al., 2017).

Inducible nitric oxide synthase (iNOS) is traditionally thought to be a central marker of classical macrophage activation. This enzyme converts the amino acid L-arginine into L-citrulline and the free radical NO (Mosser & Edwards, 2008). During physiological conditions, NO is produced by neuronal and endothelial nitric oxide synthases (nNOS and eNOS, respectively), acting as a short-lived vasodilator and a short-range signaling molecule. In inflammation, *INOS* is induced to produce large

amounts of NO, mobilizing its direct cytotoxic effects against invading pathogens (which can also be deleterious to host tissues) (Knowles & Moncada, 1994, Moilanen et al., 1999). The role of iNOS and NO in murine macrophages is well established, but *INOS* expression in human macrophages is more controversial, as *INOS* expression hasn't been successfully demonstrated in many "resting state" human macrophage populations. However, human macrophages from inflamed tissues (such as lung with tuberculosis, atherosclerotic plaques and inflammatory tissue around aseptically loosened joint prostheses) have been shown to robustly express *INOS*, demonstrating the proinflammatory role of this factor also in humans (Kieler et al., 2021, Moilanen et al., 1997).

Classically activated (M1) macrophages produce a variety of proinflammatory cytokines, most important of which are thought to be IL-1, IL-6, TNFα and monocyte chemotactic protein 1 (MCP-1, also known as chemokine C-C motif ligand 2 or CCL2). Compared to the short-lived NO, these mediators can act also in a systemic manner, directing the inflammatory response throughout the body. IL-1 induces proliferation and activation of neutrophils, lymphocytes (both B and T cells) and macrophages. It also promotes TNF production by other cells (Dinarello, 2011). IL-6 stimulates neutrophils and lymphocytes while suppressing anti-inflammatory regulatory T cells. It also activates the synthesis of acute phase proteins in the liver. In addition, IL-1 has anti-inflammatory properties via induction of IL-1 receptor antagonist (IL-1RA) and IL-10 (probably as a kind of endogenous negative feedback loop) (Abbas et al., 2018c). TNFα also promotes neutrophil proliferation and migration into tissues, activates acute-phase protein synthesis, and supports classical macrophage activation (Sedger & McDermott, 2014). MCP-1 is a chemokine (factor promoting cell movement, i.e. chemotaxis). It attracts monocytes, T cells and dendritic cells to the site of inflammation. Proinflammatory cytokines, especially IL-1 and TNFα, also act as *endogenous pyrogens*, promoting prostaglandin E_2 (PGE_2) synthesis in the hypothalamus and thus raising body temperature (Abbas et al., 2018c).

Classically activated macrophages in disease

Despite its central role in fighting bacterial infections, excessive or dysregulated classical macrophage activation has been linked to various disease states involving chronic inflammation. These include the well-known autoimmune disease rheumatoid arthritis (Angelotti et al., 2017; Wang et al., 2017), in which radiological

joint damage has been shown to correlate with the degree of macrophage infiltration into the joint (Udalova et al., 2016). These macrophages are heavily skewed towards the classical (M1) phenotype as determined by comparing the expression levels of central M1 and M2 markers (Kinne et al., 2007). These cells promote inflammation by secreting proinflammatory mediators and produce ECM-degrading MMPs but also their inhibitor TIMP-1 (Newby et al., 2016). They can also present autoantigens to T cells, perpetuating the autoimmune process characterizing RA (Ireland & Unanue, 2011). The effectiveness of several precisely targeted anti-inflammatory medications such as TNFα blockers (targeting a cytokine produced mainly by M1 macrophages) in autoimmune diseases such as RA provides further evidence for the central role of macrophages in these disorders (Kievit et al., 2007).

Macrophages also contribute to the more subtle chronic inflammation present in OA, and significant macrophage presence has been detected in up to 90% of end-stage knee OA synovial samples (Hügle et al., 2016). Like in RA, most of these macrophages are polarized in the classical (proinflammatory) direction (Smith et al., 1997). The proinflammatory factor CCL2/MCP-1, a cytokine that attracts macrophages to inflamed tissue, has been studied extensively in OA, and its levels have been shown to correlate with pain and disability in OA patients (Li & Jiang, 2015; Scanzello, 2017). Another factor potentially driving classical macrophage activation in OA are damage-associated molecular patterns (DAMPs); tissue debris released by processes such as trauma or earlier cartilage degradation. This debris contains fragments of glycosaminoglycans (GAGs) such as hyaluronan (Stabler et al., 2017) as well as glycoproteins such as tenascin-C (Sofat et al., 2012), which have been shown to promote inflammation and cartilage degradation. Bone-derived hydroxyapatite crystals are widely found in OA cartilage samples and correlate with the severity of the disease (Fuerst et al., 2009; Gibilisco et al., 1985). These crystals may (classically) activate macrophages via the inflammasome pathway, leading to release of IL-1β and IL-18, as well as increased production of catabolic enzymes and suppression of ECM synthesis (Jin et al., 2011). Basic calcium phosphate (BCP) crystals, also often present in OA joints, can also activate macrophages via activation of spleen tyrosine kinase (Syk), phosphoinositide 3-kinase (PI3K) and MAPK pathways, and the production of the alarmin S100A8 (Corr et al., 2017). Obesity has been shown to skew the predominant phenotype of macrophages towards M1 activation and to promote their migration into joints, causing development of OA-like tissue changes (Sun et al., 2017).

Interventions directed at macrophages have shown efficacy in models of OA. For example, deletion of CCL2/MCP-1 or its receptor C-C chemokine receptor type 2 (CCR2) reduces joint infiltration of monocytes and ameliorates cartilage destruction in experimental OA (Raghu et al., 2017). Also, depleting synovial macrophages reduces osteophyte formation and levels of osteogenic growth factors in collagenase-induced arthritis (Blom et al., 2004).

Recently, much attention has been paid to the involvement of macrophages in pathological states involving "silent" low-grade inflammation. These include many "diseases of affluence" including osteoarthritis (H. Zhang et al., 2018), atherosclerosis (Colin et al., 2014), obesity, nonalcoholic fatty liver disease (Alisi et al., 2017) and insulin resistance (Lauterbach & Wunderlich, 2017). A central feature of these states seems to be derangement of adipose tissue macrophage phenotype (Lin & Wei, 2017), with the predominant phenotype switching to M1, along with a constant activation of the proinflammatory c-Jun N-terminal kinase (JNK) and NF-κB pathways (Johnson & Olefsky, 2013; Piya et al., 2013). At least some of these changes may be caused directly by high levels of circulating fatty acids, which may alter, for example, peroxisome proliferator-activated receptor gamma (PPARγ) activation (see p. 33) (Kalupahana et al., 2011). Increased fat accumulation may cause hypoxia in adipocytes, which leads to proinflammatory response (Kusminski et al., 2016). Also, saturated fatty acids may cause (some of) their harmful effects via TLR4-mediated M1 activation (Hennessy et al., 2010).

2.2.3 Alternative macrophage activation

Macrophages whose net effects are anti- rather than proinflammatory are collectively called alternatively activated (M2) macrophages. These cells regulate inflammation, limiting damage caused to the surrounding tissues. They also participate in the resolution stage of inflammation and repair of tissue damage. (Hume, 2015; Male et al., 2021) "Wound-healing" alternatively activated macrophages induced by IL-4 and IL-13 produce ECM precursors from arginine via ornithine (Comalada, Yeramian et al., 2012), and also direct the actions of other cell types such as fibroblasts and myofibroblasts by producing profibrotic growth factors and other mediators (Sindrilaru & Scharffetter-Kochanek, 2013).

Recently, several authors have pointed out that the nomenclature concerning alternative macrophage activation has become confusing and inconsistent. This

difficulty is compounded by the fact that several different stimuli can be used to induce an anti-inflammatory macrophage phenotype. These include IL-4 and IL-13, glucocorticoids and immune complexes. Additionally, alternative activation markers are often at least partly specific to certain species. While human macrophages display an alternative phenotype induced by IL-4 and IL-13, the markers of this phenotype are somewhat distinct from those of murine M2 cells. For example, human macrophages do not express *Ym1* (also known as chitinase-like 3 or *Chil3*) or *Fizz1* (also known as resistin like alpha or *Retnla*), both widely used murine M2 markers (Raes et al., 2005), and robustly express arginase 1 (*Argl*) only under special conditions such as trauma or stimulation with certain immunostimulatory compounds (Ochoa et al., 2001; Rouzaut et al., 1999). Instead, alternative activation markers in human cells include CCL13 and PDGFB (Martinez et al., 2006; Song et al., 2000).

Mechanisms of alternative activation

T_H2 cytokines IL-4 and IL-13 are canonical inducers of alternative macrophage activation (Martinez et al., 2009). IL-4 induces the differentiation of T_H2 cells, which in turn produce more of this cytokine in a positive feedback loop. Basophils are thought to initiate the process by producing the first dose of IL-4 (Sokol et al., 2008). IL-13 is produced by many different types of cells, including T_H2 cells, macrophages and natural killer (NK) cells, mast cells, eosinophils and basophils (Rael & Lockey, 2011). IL-4 and IL-13 signal through a shared pathway, a heterodimer receptor complex composed of alpha IL-4 receptor (IL-4Rα) and alpha IL-13 receptor (IL-13R1), the activation of which in turn activates insulin receptor substrates (IRS) 1 and 2, as well as STAT6 (Junttila, 2018), which regulates the expression of several M2 marker genes (Martinez et al., 2009). IL-13 also binds to the alpha 2 IL-13 receptor (IL-13Rα2), a "decoy" receptor that appears to sequester the cytokine and counteract T_H2 responses (Kasaian et al., 2011).

Of the signal transducer and activator of transcription (STAT) family of transcription factors, STAT3 is also capable of inducing M2 activation. It seems to function in an autocrine-paracrine manner: for example, *mycobacterium tuberculosis* infection activates TLR-dependent Myd88, followed by CCAAT-enhancer-binding protein beta (C/EBPβ) activation and production of M2-stimulating cytokines such as IL-10, which act via STAT3 to promote the expression of M2 factors (Qualls et al., 2010).

Another important signaling pathway for alternative macrophage activation is the phosphoinositide 3-kinase **(**PI3K**) /** Akt (also known as protein kinase B) pathway. Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase (SHIP) antagonizes this pathway, and macrophages deficient in it are biased toward the alternative activation (Qin et al., 2020).

Peroxisome proliferator activated receptors (PPARs) also seem to play an important role in the alternative macrophage activation (Chawla, 2010; C. Li et al., 2017). They have been postulated to promote alternative macrophage activation especially in adipose tissue and in healing wounds, where the role of IL-4 and IL-13 is less clear (Gordon & Martinez, 2010). Specifically, PPARγ, in addition to attenuating inflammation by antagonizing the transcription factors NF-κB and AP-1 (Ricote et al., 1998), also seems to "shift" macrophage activation from classical towards the alternative phenotype (Huang et al., 1999). This is evidenced by the fact that mice deficient in PPARγ display impaired alternative macrophage activation and are more susceptible to obesity and insulin resistance (Odegaard et al., 2007). In contrast, PPARα activation appears to reduce alternative macrophage activation (Paukkeri et al., 2015). PPARγ exists as two isoforms, of which PPARγ1 is expressed in most tissues (including macrophages) whereas PPARγ2 is mostly found in adipose tissue and the intestine (Fajas et al., 1997; Lefterova et al., 2010). PPARγ is activated by various endogenous ligands such as polyunsaturated fatty acids and their metabolites, and several of these metabolites (including eicosanoids) are involved in inflammation, highlighting yet another link between metabolism and inflammation. IL-4 promotes M2 activation via both increasing PPARγ expression in macrophages and by stimulating cellular production of its ligands via the 12/15-lipoxygenase pathway (Rigamonti et al., 2008). Intriguingly, there is also evidence of cooperation between STAT6 and PPARγ in promoting alternative macrophage activation, with the former acting as a cofactor for the latter (Szanto et al., 2010).

cAMP response element-binding protein – CCAAT–enhancer-binding protein (CREB – C/EBP) pathways appear to play a central role in the regulation of both classical and alternative macrophage activation (Polumuri et al., 2021). C/EBPs are transcription factors that bind to the promoter regions of several genes, thus inducing their transcription. C/EBPβ has been shown to mediate the expression of several known alternative activation markers, including arginase 1 and Ym-1. C/EBPβ is itself induced by transcription factor cAMP-responsive element-binding protein (CREB), and deletion of the CREB-binding site in its promoter region

impairs alternative macrophage activation and muscle tissue repair (Ruffell et al., 2009).

Interferon regulatory factors (IRFs) bind to the so-called interferon consensus sequence located upstream of interferon genes, regulating their transcription. As various interferons have been shown to modulate macrophage phenotypes (S. Huang et al., 2016; C. Li et al., 2017; Moreira-Teixeira et al., 2016), it is fitting that IRFs are also implicated in this role. IRFs 1, 2 and 5 promote classical macrophage activation (Salkowski et al., 1999). IRF5 also downregulates many alternative activation markers (Krausgruber et al., 2011), and IRF6 inhibits alternative activation by downregulating PPARγ (C. Li et al., 2017). Conversely, IRF3 promotes alternative macrophage activation by activating the PI3K/Akt pathway (Tarassishin et al., 2011), and IRF4 deficiency reduces the expression of a large number of M2 markers (El Chartouni & Schwarzfischer, 2010).

Markers of alternative activation

The "canonical" marker of alternative activation is arginase 1 (ArgI). This is the cytosolic isozyme of arginase, the other being the mitochondrial isozyme ArgII. In the liver, arginase is the final enzyme of the urea cycle, converting the amino acid arginine to ornithine and urea and therefore participates in the clearance of otherwise harmful amines from the body. In macrophages, ArgI is thought to regulate inflammation and promote resolution via at least two distinct mechanisms: first, it starves iNOS (see p. 28) of its arginine substrate, and second, ornithine can be converted to proline and polyamines, precursors of ECM components such as collagen (Krane, 2008, Male et al., 2021).

IL-4-induced *ArgI* expression is mediated mainly via the transcription factors STAT6 and C/EBPβ (Pauleau et al., 2004). IL-4 causes them to bind the *ArgI* gene 3 kb upstream of the transcription start site, initiating the transcription (Gray et al., 2005). STAT6 also promotes *ArgI* expression via epigenetic changes, namely methylation and jumonji domain containing 3 (Jmjd3) -dependent demethylation of different lysine residues of histone 3 (Ishii et al., 2009). Krüppel-like factor 4 (KLF4) is also induced by IL-4 but seems to be unable to promote *ArgI* expression by itself. However, STAT6 promotes the binding of KLF4 to the aforementioned response element in the *ArgI* gene, facilitating its action (Liao et al., 2011).

Nuclear receptors, especially PPARs (see p. 33), are necessary for *ArgI* expression. Both PPARδ (Odegaard et al., 2008) and PPARγ (Odegaard et al., 2007) bind to peroxisome proliferator response elements in the promoter of the gene as heterodimers with retinoid X receptor (RXR) (Nagy et al., 2012). This heterodimer has been called "permissive", since both PPAR and RXR ligands can activate it (Gallardo-Soler et al., 2008). IL-4 also induces the production of natural PPARγ ligands in macrophages, providing yet another mechanism for *ArgI* induction caused by this cytokine (Huang et al., 1999).

Liver X receptors (LXRs) are another family of nuclear receptors central for *ArgI* expression. Instead of directly binding *ArgI* promoter, LXRα promotes interaction between the transcription factors PU.1 and IRF8, which then bind *ArgI* promoter and activate its transcription (Pourcet et al., 2011). In contrast to PPARs, however, no production of endogenous LXR ligands in IL-4-stimulated macrophages has been described (Pourcet & Pineda-Torra, 2013). Vitamin D receptor (VDR) also promotes *ArgI* expression (Ehrchen et al., 2007). The exact mechanism is yet to be elucidated. However, VDR has been shown to promote *IRF8* expression (Ramagopalan et al., 2010), and thus it might act in a manner analogous to LXRα.

The role of *ArgI* in human, compared to murine, macrophages is less clear, as the T_H2 cytokine IL-4 alone does not seem to induce its expression in peripheral blood monocytes or monocyte-derived macrophages (Thomas & Mattila, 2014). However, increased *ArgI* expression has been detected in terminally differentiated alternatively activated macrophages in disease states such as atherosclerotic plagues (Yurdagul et al., 2020). Also, cotreatment of human macrophages with IL-4 and the PDE4 inhibitors rolipram markedly induces *ArgI*, underlining the role of secondary signaling pathways in the process (Erdely et al., 2006). It is yet partly unclear how these findings translate to conditions present in the body during disease processes.

The anti-inflammatory phosphatase mitogen-activated protein kinase phosphatase 1 (MKP-1) has also been implicated to play a role in alternative macrophage activation. Deficiency of this gene has been shown to bias macrophages towards stronger classical activation and increased production of proinflammatory mediators (Korhonen & Moilanen, 2014). There are also some results suggesting that MKP-1 deficiency may decrease the expression of alternative activation markers while increasing that of classical ones (Kim et al., 2016).

Tissue healing processes promoted by alternatively activated macrophages are mediated by a variety of growth factors that stimulate the functions of other cell types in the environment. These include platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) and fibroblast growth factor 2 (FGF2) (Shapouri-Moghaddam et al., 2018). PDGF plays a central role in several processes related to tissue growth, including angiogenesis and proliferation of fibroblasts, smooth muscle cells and mesenchymal stem cells (Papadopoulos & Lennartson, 2018). It also appears to mediate the profibrotic effects of alternatively activated macrophages in both physiological conditions and diseases such as SSc (Trojanowska, 2008; Wynn et al., 2013).

The TGF-β family consists of four different isoforms (TGF-β 1 through 4). These multifunctional cytokines promote fibroblast differentiation and proliferation and production of ECM components like collagen I (Meng et al., 2016). They also promote T cell proliferation and differentiation; interestingly, this includes both anti-inflammatory T_{reg} cells and proinflammatory T_H17 ones (S. Zhang, 2018).

FGF2 is a mitogenic and survival-promoting growth factor (Coleman et al., 2014). It has also been linked to angiogenesis in wound healing, a function that is in line with this important role of alternatively activated macrophages (Liu et al., 2013).

The mannose receptor (MRC1, also known as CD206) is a C-type lectin (carbohydrate binding protein). This pattern recognition receptor (PRR) recognizes mannose, N-acetylglucosamine and fucose residues present in glycan modifications of proteins (Schlesinger et al., 1978), leading to clathrin-mediated endocytosis of those proteins. As many micro-organisms carry such structures on their surface, this can be employed for immune response; cells bearing MRC1 can take up glycosylated peptides released from the surface of microbes and present them to T cells, thus acting as parts of adaptive immunity (Burgdorf et al., 2006). During the resolution phase of inflammation, MRC1-mediated endocytosis is employed to remove excess proinflammatory glycoproteins (such as lysosomal hydrolases, tissue plasminogen activator and myeloperoxidase) from the tissue (Gazi & Martinez-Pomares, 2009). Similarly, in physiological conditions, MRC1 facilitates recycling of circulating glycoproteins (Lee et al., 2002).

Mannose receptor itself does not possess capability for intracellular signaling, and, in contrast to many other endocytosis-mediating receptors, does not seem to promote inflammation in itself (Stahl & Ezekowitz, 1998). However, there is some

evidence that it might facilitate production of cytokines such as IL-8 when activated in conjunction with certain other receptors (TLR2 in the case of IL-8) (Tachado et al., 2007). Intriguingly, polymorphism in the MRC1 gene have been linked to susceptibility to asthma (Hattori et al., 2009) and sarcoidosis (Hattori et al., 2010), though mechanisms of these associations remain unknown.

Ym-1 is an established alternative macrophage activation marker in murine cells (Chang et al., 2001). It is structurally related to chitinases (enzymes that break down chitin, an essential structural component of some fungi and invertebrates), but lacks chitinase activity (Nair et al., 2005). It, however, binds chitin and acts as a chemotactic factor for T cells and eosinophils, thus promoting immune response against nematode infections (Zhao et al., 2013).

Fizz1 is likewise an antiparasite factor, secreted by alternatively activated macrophages in response to helminth infection (Raes et al., 2002). This protein also seems to have a role in tissue repair, as it has been found to promote differentiation of, and collagen production by, myofibroblasts (Liu et al., 2004).

Finding a robust alternative activation marker in human macrophages has proven relatively elusive. CCL13 is a T_H2 chemokine that promotes the chemotaxis of various immune cells such as macrophages, T lymphocytes and eosinophils, and it has been implicated to play a role in allergic reactions and asthma (Lamkhioued et al., 2000). It also has been shown to be markedly upregulated in IL-4-stimulated human macrophages (Martinez et al., 2006). This is corroborated by the finding that the levels of this cytokine are increased in several pathological states involving alternative macrophage activation. These include atopic dermatitis (Taha et al., 2000), asthma (Taha et al., 2001) and systemic sclerosis (Yanaba et al., 2010). Other chemokines identified as human alternative activation markers in transcriptomic studies include CCL18 and CCL23 (Martinez et al., 2006).

Alternatively activated macrophages in disease

In the setting of inflammation, alternatively activated macrophages protect the body from harmful, excessive inflammation and support tissue healing. However, disproportionate or dysregulated alternative activation has also been linked to pathologic conditions. Most of these processes involve excessive growth of tissues, and thus can be thought of as derangements of normal tissue repair processes. This

includes several fibrosing diseases (see p. 50) such as pulmonary fibrosis (Murthy et al., 2015; Tao et al., 2014), chronic pancreatitis (Xue et al., 2015) and systemic sclerosis (SSc) (Maier et al., 2017; Yamashita et al., 2017).

Macrophage infiltration is a feature of SSc, and generation of profibrotic mediators by alternatively activated macrophages is a plausible pathogenetic mechanism. This has been confirmed by studying the activation signatures of macrophages isolated from skin lesions of SSc patients (Higashi-Kuwata et al., 2010). The issue of macrophage polarization in fibrosis is not, however, quite straightforward. Studies of both dermal (Raker et al., 2017) and lung (Williamson et al., 2015) fibrosis show at least part of the macrophages to express also proinflammatory mediators such as IL-1β and TNFα, as well as MMP enzymes traditionally linked with proinflammatory classical activation (Newby, 2016). Thus, macrophages associated with fibrotic lesions may include groups of classically and alternatively activated cells with their proportions depending on the age and state of the disease.

2.3 Joint structure, synovium and cartilage

Synovial (or diarthrodial) joints are structures connecting two bones together, providing structural support while enabling movement. They consist of a fibrous joint capsule that is continuous with the periosteum of joined bones, synovial membrane lining the inner part of the joint cavity as well as hyaline cartilage covering the articulating surfaces of the bones. (Scanzello & Goldring, 2021)

2.3.1 Synovium

Synovium, the tissue lining the joint cavity, is a thin layer between the outer, fibrous joint capsule and the fluid-filled synovial space. It produces synovial fluid that provides nutrition for the articular cartilage and lubricates the joint surfaces. The synovium is further divided into functional compartments: the lining region (synovial intima), the subintimal stroma and the neurovasculature. The synovial intima contains two cell populations: 10-20% of synovial cells are specialized macrophages (type A synovial cells) and 80-90% specialized fibroblasts (type B synovial cells). (Iwanaga et al., 2000; Veale & Firestein, 2021)

Type A synoviocytes express cluster of differentiation (CD) 163 and 97 as well cathepsins B, L, and D. A subpopulation of type A synoviocytes also expresses CD16, which is a surface marker specific to certain tissue macrophages. Type B synoviocytes are marked by the expression of vascular cell adhesion molecule-1 (VCAM-1), cadherin-11, TLR2 and uridine diphosphoglucose dehydrogenase (UDPGD). Type A synoviocytes are phagocytotic and may participate in the cleaning of particulate matter from the joint cavity. Their origins are subject to some controversy: type A synovial cells can be found in embryonic synovium, suggesting that they are "seeded" in the tissue during development like many other types of tissue macrophages (Carvalho de Moraes et al., 2015). On the other hand, there are several lines of evidence suggesting that type A synovial cells are recruited from bone marrow -derived circulating monocytes (Veale & Firestein, 2021). It may be that the population of type A synoviocytes in a joint includes both embryonic and bone marrow -derived cells (Tu et al., 2019). During inflammation, additional monocytes are recruited from the circulation to the synovium, where they differentiate into macrophages.

Fibroblast-like type B synoviocytes have ample protein synthesis capability; they produce several ECM and synovial fluid components such as collagens, sulfated proteoglycans, fibronectin, tenascin, hyaluronan and lubricin. The subintimal layer contains undifferentiated macrophages. It has also been shown to contain its own fibroblast population which has been shown to be phenotypically distinct from type B synovial cells. This cell population is marked by expression of CD90 and CD248. (Ospelt, 2017; Veale & Firestein, 2021)

2.3.2 Hyaline cartilage

Hyaline cartilage is a resilient, elastic and low-friction tissue that is largely responsible for the mechanical properties of synovial joints. Hyaline cartilage matrix consists of proteins (especially type II collagen), proteoglycans (such as aggrecan), glycosaminoglycans (such as hyaluronan and chondroitin sulfate, usually as constituents of proteoglycans) and water (with water content of up to 80% being reported in articular cartilage) (Shiguetomi-Medina et al., 2017). Embedded in the matrix, in well-defined niches called lacunae, are chondrocytes, cells of the cartilage, which account for only 1-2% of the total volume of normal cartilage. Having no nerves or blood vessels, cartilage obtains nutrients via diffusion from the synovial fluid. (Erggelet & Mandelbaum, 2008; Mobasheri et al., 2021).

Cartilage is divided into four layers, or zones, separated in depth. These are the superficial tangential ("gliding") zone, the middle ("transitional") zone, the deep ("radial") zone and the calcified cartilage zone. The border between the non-calcified and calcified cartilage layers (zones 3 and 4) is called the tidemark. Chondrocyte density progressively decreases from the superficial towards the deep part of cartilage. Chondrocyte size and morphology also differ between the layers: cells in the superficial zone are relatively small and elongated, while those in the deeper parts of the cartilage are larger and more spherical. The superficial zone contains thin collagen fibrils arranged tangentially to the surface, while the collagen fibrils in the middle zone are larger and arranged radially. The deep zone contains a larger amount of aggrecan relative to collagen compared with the more superficial zones. Relative water content decreases with depth. (Mobasheri et al. 2021)

In addition to collagen II, cartilage also contains several minor collagen components such as collagens IX and XI (which are specific to cartilage) as well as VI, XII, and XIV (which are found in several connective tissue types). The other (apart from collagens) major group of cartilage ECM constituents are proteoglycans, macromolecules consisting of a protein core and numerous complex carbohydrate chains attached to it. The most abundant proteoglycan in articular cartilage is the large aggregating proteoglycan aggrecan. It forms large complexes with the glycosaminoglycan hyaluronan (linked with the so-called link protein); these complexes may contain up to 100 aggrecan units. Several other proteoglycans, such as versican, perlecan, decorin and lumican, are present in smaller quantities. Proteoglycan 4 (PRG4), also known as superficial zone protein (SZP) or lubricin, is an important proteoglycan produced mainly by chondrocytes located superficially in the cartilage. It lubricates the cartilage surface and contributes to the elastic properties of the synovial fluid. Cartilage protein turnover is exceptionally slow; the half-life of aggrecan core protein has been estimated as 25 years (even though some of its subunits, such as the G3 globular domain, may decay much faster) and that of collagen II as up to 100 years. (Hsueh et al., 2019; Mobasheri et al., 2021) Chondrocytes and cartilage ECM exist in a complex mutual relationship: chondrocytes synthesize ECM and support its integrity, and ECM components in turn affect chondrocyte differentiation and function via surface receptors such as integrins, discoidin domain receptor 2 (DDR 2) and CD44 (Prein & Beier, 2019).

Cartilage is an avascular, hypoxic environment, and thus it has a limited capacity for repair and regeneration. The chondrocytes are also fixed in place in lacunae, and thus

cannot migrate to a site of damage, while dearth of oxygen and nutrients limits their synthetic functions. When cartilage is damaged by trauma or degenerative changes (such as osteoarthritis), chondrocytes upregulate the production of ECM components to undo part of the damage. Eventually, damaged cartilage may be (partially) replaced by fibrous scar tissue resembling fibrocartilage found in some nonsynovial joints. This tissue contains type I collagen in addition to type II found in hyaline cartilage, and has inferior mechanical properties. (Deng et al., 2018; van der Kraan, 2017)

2.3.3 Chondrocytes

Chondrocytes are derived from mesenchymal stem cells (MSCs), a process that is regulated by factors such as FGF2, bone morphogenetic protein 4 (BMP4), Wnt signaling and TGFβ (Lee et al., 2013). Chondrocytes play a central role in endochondral ossification, one of the two processes (along with intramembranous ossification) by which bone is created during fetal development. During ossification chondrocytes proliferate and adopt a so-called hypertrophic phenotype, marked by increased cell volume and synthesis of ECM components. In mature, healthy cartilage, chondrocytes maintain cartilage structure by secreting matrix constituents. They maintain a balance between ana- and catabolic processes, resulting in a steady turnover of cartilage constituents. As stated before, type II collagen and aggrecan core protein exhibit very slow turnover, whereas the GAG parts of aggrecan are replaced more often. Other cartilage matrix components produced by chondrocytes under low-turnover conditions include biglycan, decorin, COMP, tenascins, and matrilins. Mature chondrocytes resist proliferation and generally do not divide. (Mobasheri et al., 2021, van der Kraan & van der Berg, 2012)

As stated before, chondrocytes reside in an avascular environment with variable oxygen availability (the difference between superficial and deep parts being more than tenfold). Thus, they are well-adapted to variable hypoxic conditions. Chondrocytes obtain nutrients from the synovial fluid at the articular surface and subchondral bone via diffusion. They also rely more on glycolysis than mitochondrial respiration for energy production, and their oxygen consumption per cell is only 2-5% of that in liver or kidney cells. Hypoxia-inducible factor 1 alpha (HIF1α) is a central "survival factor" that facilitates chondrocyte metabolism in hypoxic conditions, increasing the expression of aggrecan and type II collagen via Sox9 (Amarilio et al., 2007; Mobasheri et al., 2021).

2.4 Osteoarthritis

Osteoarthritis is the most common form of arthritis and a central example of a disease associated with chronic inflammation (Mathiessen & Conaghan, 2017). The disease is marked by low-grade inflammation associated with destruction of the articular cartilage resulting in narrowing of joint spaces, transient exacerbations of synovial inflammation and formation of osteophytes (bony overgrowths) along joint margins. These changes cause pain, stiffness and loss of mobility; OA is a leading cause of disability in the elderly. In a clinical setting, OA is generally diagnosed on the basis of distinctive symptoms and X-ray findings. (Glyn-Jones et al., 2015)

Epidemiology and risk factors of OA

Estimates of OA prevalence depend greatly on the precise definition and methodology (whether clinical or radiographic findings were assessed, etc.). However, it has been estimated to affect over half of the population over 55 years of age (Hunter & Bierma-Zeinstra, 2019). Of the Finnish people aged 65-74, about 12 % has been estimated to have symptomatic hip OA, while the prevalence of knee OA was estimated as 11 % among males and 18 among females in the same age group (Arokoski et al., 2007). Early OA is thought to be underdiagnosed especially among younger patients, and some studies have found that over 25% of people over 45 years of age have symptoms suggestive of OA (AIHW, 2020). Regardless of the precise prevalence, OA undoubtedly causes widespread disability and large costs to healthcare systems. In addition to age, other major risk factors include female gender, obesity, joint trauma and genetic factors (O'Neill et al., 2018). According to radiographic data, small joints in the hand are most commonly affected by OA. In the lower limbs, the hip and knee joints are most likely to be involved. Other joints commonly affected by OA include facet joints in the spine and the temporomandibular joint; in theory, any joint in the body can be affected (Glyn-Jones et al., 2015; Nelson, 2021).

Age is the most important risk factor for OA, which probably reflects the accumulation of tissue damage, loss (by apoptosis) of chondrocytes and the reduced ability of surviving chondrocytes to synthesize proteins that are essential for joint function. Obesity greatly increases OA risk, probably via both increased mechanical loading as well as increased production of proinflammatory cyto- and adipokines by adipose tissue. Trauma is another central risk factor for OA; this covers both singular

severe instances of trauma (such as intra-articular fractures) as well as subtle, repetitive trauma such as that caused by some sports or the use of power tools. Gender also affects OA risk, and hormonal factors are implicated in this. While premenopausal women are less likely to develop OA than men of comparable age (possibly owing to higher incidence of injuries in men), this is reversed after the menopause, and overall women are twice as likely as men to develop OA. Chondrocytes express estrogen receptors, and loss of estrogen-induced cartilage homeostasis is thought to promote OA development in postmenopausal women; fittingly, estrogen replacement therapy has been shown to reduce OA risk in postmenopausal women (Di Cesare et al., 2021). Genetics of OA have been investigated in several genome-wide association studies (GWASs), and these have identified several dozes of genes associated with OA (Aubourg et al., 2021; Evangelou et al., 2014; Skarp et al., 2018; Wang et al., 2016; Zeggini et al., 2012). These genes include, among others, the cartilage-specific collagen *COL11A1*, proinflammatory factors such as cyclo-oxygenase 2 (*COX2*) and *IL6* as well as growth factors such as growth differentiation factor 5 (*GDF5*) and vascular endothelial growth factor A (*VEGFA*). (Aubourg et al., 2021; Skarp et al., 2018). OA development is also linked to inflammatory arthritides such as gout and RA. Systemic and local inflammatory states observed in these disorders may provoke cartilage catabolism and impair its healing, eventually leading to the development of OA (Lee et al., 2020). RA may also impair joint stability, adding to mechanic stresses that predispose to OA (Wang et al., 2019).

Recently, a concept of several distinct OA phenotypes has emerged. According to one well-established scheme, these include ageing-driven, cartilage-driven, traumatic injury-driven, subchondral bone -driven, synovitis-driven inflammatory, and metabolic phenotypes (Mobasheri et al., 2017). These phenotypes are hypothesized to display different (though naturally overlapping) features and pathological processes.

Pathophysiology of OA

Osteoarthritis was once thought of as a pure "wear and tear" disease caused by mechanical cartilage degradation. However, nowadays OA is regarded as a multifaceted pathological process affecting all joint tissues, and multiple factors (such as cytokines, mechanical micro- or macrotrauma and genetic predisposition) are involved in its pathogenesis. The chronic inflammatory component of OA has

become increasingly appreciated during the last few decades (Scanzello, 2017), and using modern imaging modalities, synovitis (inflammation of the synovial membrane) can be detected in a majority of patients (Mathiessen & Conaghan, 2017). Constant low-grade inflammation in the joint contributes to pain, increased catabolism and degradation of articular cartilage (Robinson et al., 2016). More overt inflammationary exacerbations ("flares") are a clinically well-known but relatively understudied phenomenon in OA. These resemble, to at least some extent, other forms of acute arthritis; they are associated with increased pain and various other signs of joint inflammation including effusion, heat and reduced range of motion (Parry et al.,.2018; Thomas & Neogi, 2020).

On the level of cartilage, OA pathogenesis involves several interconnected alterations in cellular and tissue function (Figure 2-2). The process is thought to be initiated by molecular ECM fragments liberated from cartilage by injury or degeneration. These fragments act as DAMPs (see p. 23), promoting inflammation and unbalancing the ana- and catabolic processes in the joint (Robinson et al., 2016). Chondrocytes, cells of the cartilage, reside within the cartilage matrix in niches known as lacunae. In OA, an increasing number of chondrocytes are lost to necrosis and apoptosis driven by abnormal mechanical and biochemical factors. This process is characterized by oxidative and nitrosative stress resulting from nitric oxide (NO) and other ROS/RNS, mitochondrial dysfunction and changes in cell-matrix interactions (Di Cesare et al., 2021; Kühn et al., 2004). In the early phases of the disease, resulting cartilage hypocellularity is compensated by proliferation of the surviving chondrocytes. These cells clonally proliferate within their lacunae, forming clusters of up to 50 cells. They secrete high amounts of cartilage matrix components such as collagen II and aggrecan, and some chondrocytes assume partially stem cell -like or hypertrophied phenotypes. This leads to increased protection of cartilage extracellular matrix (ECM) components; however, the mechanical quality of the resulting ECM appears to be inferior compared to healthy cartilage (Di Cesare et al., 2021).

In addition to chondrocytes, other cell types have been shown to play important roles in OA pathogenesis. Fitting with the inflammatory nature of OA, central regulators of inflammation, macrophages and T_H cells, have been detected in OA synovium (in addition to type A and B synovial cells). These cells display phenotypic heterogeneity, probably reflecting balance between proinflammatory and anti-inflammatory "feedback" mechanisms. For T_H cells, the proinflammatory T_H1 and

T_H17 phenotypes dominate (Y. Li et al., 2017), but anti-inflammatory T_H2 cells have also been detected in OA synovium (Nees et al., 2020). Likewise, both macrophages displaying classical (M1) (H. Zhang et al., 2018) and alternative (M2) markers (Wu et al., 2020) (see p. 25) have been shown to be present.

OA is characterized by an altered balance between catabolic and anabolic (reparative) processes in the cartilage. Inflammatory mediators such as IL-1 and tumor necrosis factor alpha (TNFα) induce chondrocytes to produce ECM-degrading proteinases such as matrix metalloproteinases (MMPs). The MMPs 1, 3 and 13 are especially implicated in OA, as they are able to efficiently degrade important cartilage constituents such as aggrecan core protein and collagen II. Other central cartilage-degrading enzymes in OA are cathepsin K as well as a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzymes 4 and 5. These proteolytic enzymes are counteracted and regulated by proteinase inhibitors such as tissue inhibitor of metalloproteinases (TIMP) enzymes and plasminogen activator inhibitor 1 (PAI-1). Plasmin, best known for its role in blood coagulation, also efficiently activates many MMPs, and its inhibition by PAI-1 is therefore chondroprotective. (Di Cesare et al., 2021)

In addition to cartilage catabolism and changes in bone structures of the joint (subchondral bone sclerosis and osteophyte formation), OA is increasingly recognized to cause other anatomic and histological changes in the joint. Increasing angiogenesis (blood vessel formation) in subchondral bone and bone-cartilage interface is driven by VEGFs, especially VEGF-A, and angiogenesis may contribute to osteophyte ossification. Healthy articular cartilage actively resists angiogenesis, but this process is disturbed in OA; angiogenic invasion into cartilage leads to proteoglycan loss and sclerosis (abnormal endochondral ossification) of the cartilage. OA is also associated with increased nerve growth stimulated by nerve growth factor (NGF). These newly formed nerves are sensitive to noxious stimuli produced by the ongoing catabolic and inflammatory processes, and thus NGF activity has been heavily linked with OA pain. (Hamilton et al., 2016, Miller et al., 2017)

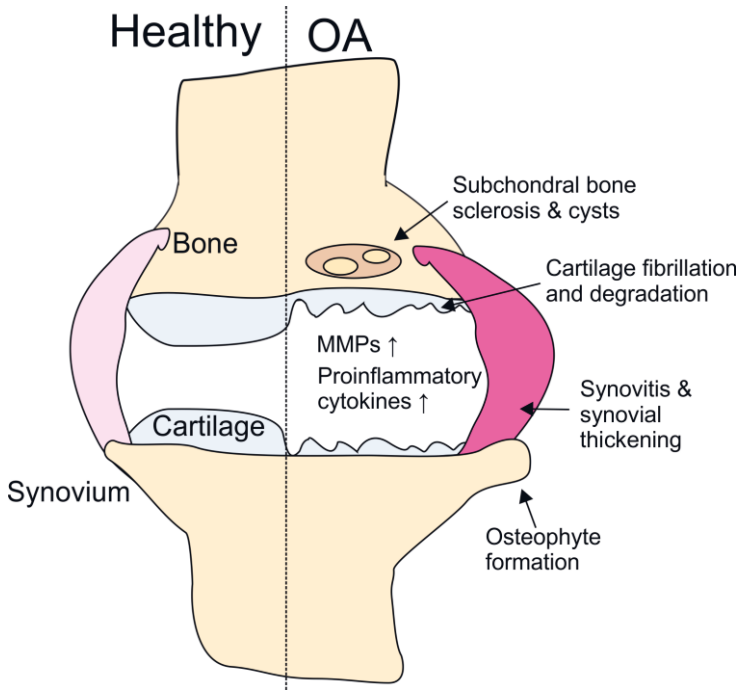

Figure 2-2. A schematic presentation of pathophysiological changes in OA involve all of the tissues in the joint. Increased amounts of catabolic MMPs and proinflammatory cytokines are observed in OA-afflicted joints. Adapted from Robinson et al., 2016.

Treatment of OA

Owing to the great suffering and costs caused by OA, there is massive interest in developing disease-modifying pharmacological therapies. Dozens of clinical trials have been conducted on this subject during the last ten years (Oo & Hunter, 2019). Explored potential treatments include inhibition of cartilage-degrading MMPs, prevention of bone loss with bisphosphonates, calcitonin or strontium, as well as blockade of various proinflammatory effectors with monoclonal antibodies and other agents. An overwhelming majority of these trials have, however, ended with failure due to concerns over either efficacy or safety, and despite intense search, there are currently no approved disease-modifying treatments in the market (Oo et al., 2021). Thus, all current pharmacological treatments, with the possible exception of glucocorticoids, are essentially symptomatic.

Currently recommended treatment modalities for OA include exercise, weight loss (in obese patients) and analgesics (paracetamol and/or NSAIDs). In severe cases,

joint replacement surgery may be indicated. Other commonly used therapies include oral glucosamine and chondroitin sulfate, as well as intra-articular hyaluronate injections. Benefits of these methods remain unclear, and likely modest at best (Train et al., 2021). In the case of glucosamine, the compound is purported to increase ECM production by acting as a precursor for GAG synthesis. However, glucosamine cannot be utilized by such, only when conjugated to uridine diphosphate (UDP). However, orally administered glucosamine (at reasonable doses) has only negligible effect on the levels of those conjugates. Glucosamine also does not affect the availability of UDP-glucuronic acid, another compound that is crucial for GAG synthesis. Due to these reasons, and supported by clinical trials, the effects of oral glucosamine on GAG synthesis can be expected to be insignificant (Qu et al., 2007).

Intra-articular injections with glucocorticoids (GCs) such as triamcinolone and methylprednisolone are another widely used treatment option in OA; they are particularly used to attenuate the inflammatory OA exacerbations (Baschant et al., 2013). Estimates of the benefits of intra-articular GCs vary, with different studies finding meaningful (Conaghan et al. 2018), slight (Jüni et al., 2015) or no (McAlindon et al., 2017) reduction in pain compared to placebo. There appears to be significant variation between responses in individual patients (Hirsch et al., 2013), which might be linked to different OA phenotypes in these people. There are also some concerns about the potentially deleterious long-term side effects of intra-articular GCs. GCs are known to promote apoptosis in a wide range of cell types (Gruver-Yates & Cidlowski, 2013), suppress tissue healing (Stojadinovic et al., 2012) and to predispose to osteoporosis (Frenkel et al., 2015). Analogously, they might well adversely affect cartilage development, homeostasis and repair. The invasive procedure also carries small but nonnegligible risks of serious complications such as septic infections (Kokubun et al., 2017). Some studies using magnetic resonance imaging (MRI) have found that regular intra-articular injections of triamcinolone acetonide accelerate the reduction of cartilage volume in the knee (McAlindon et al., 2017), while other studies have found no radiographic effects (Spitzer et al., 2019). In the light of preceding information, determining the detailed effects of intra-articular GCs is an important question in chondrocyte immunopharmacology.

2.4.1 Chondrocyte phenotype in OA

Phenotypic changes in chondrocytes have been most intensively studied in the context of OA. As stated before, OA is associated with loss of chondrocytes, and

the gene expression profile of surviving cells has been shown to be significantly altered. Some of these changes are thought to be anabolic and cartilage-protecting (such as increased synthesis of some ECM components), and some others catabolic and harmful (e.g. secretion of proteolytic enzymes and proinflammatory cytokines, altered expression of enzymes related to posttranslational modification of glycosaminoglycans) (Han et al., 2017; Sandy et al., 2015). Alterations in chondrocyte metabolism have also been linked to OA. These include increased production of ROS and RNS, mitochondrial damage as well as defective AMP activated protein kinase (AMPK) signaling (Zheng et al., 2021). Also, the major energy production mode in the cells appears to shift from oxidative phosphorylation to the much less effective anaerobic glycolysis (Zheng et al., 2021). Chondrocytes in OA cartilage also acquire some characteristics of premature senescence, a cellular state characterized by reduced/absent ability to proliferate and deranged gene expression profile. However, the potential causal link between these changes and OA is still unclear (Di Cesare et al., 2021). Genome-wide expression analyses (GWEA studies) have been performed comparing chondrocyte gene expression between degraded and preserved OA cartilage; these have employed either microarrays or next generation sequencing -based analysis (RNA-Seq). These studies have identified thousands of statistically differentially expressed genes, including those involved in cartilage development (such as *FRZB*), inflammation (such as microsomal prostaglandin E synthase-1, *PTGES* and *IL11*) and pain (such as *NGF*) (Ramos et al., 2014; Almeida et al., 2019). Even more advanced genomic techniques such as single-cell RNA sequencing are an interesting avenue of modern research in OA pathophysiology, allowing comprehensive studies of the differences in gene expression between healthy and OA chondrocytes (Wang et al. 2021).

One of the best-studied changes displayed by chondrocytes in OA is abnormal hypertrophy (increase in cell size). This is associated with upregulation of type X collagen, a canonical hypertrophy marker (Aigner et al. 1993, Little et al., 2009). As other features of endochondral ossification, such as vascularization and calfication, can also be observed in OA cartilage, the disease has been hypothesized to involve a "recapitulation" of embryonic ossification and hypertrophic chondrocyte phenotype (van der Kraan & van den Berg, 2012). As an example, osteophyte formation is thought to involve chondrogenic differentiation of MSCs, and later transformation of the resultant cartilage into bone (van der Kraan & van den Berg, 2007). As catabolism (as a mechanism of remodelling) is intimately coupled to cartilage and bone development, this might also contribute to the upregulation of

matrix-degrading enzymes in OA (Di Cesare et al., 2021; van der Kraan & van den Berg, 2012).

OA is associated with increased presence of inflammatory cytokines (such as IL-1β, IL-17, and TNFα) in the joint, and chondrocytes in OA are driven to produce further proinflammatory factors (such as IL-8, IL-6, NO and PGE_2) (Wojdasiewicz et al., 2014). Nitric oxide (NO) produced by iNOS is another central inflammatory and catabolic mediator produced by chondrocytes in OA (Vuolteenaho et al., 2001; Vuolteenaho et al., 2015). Acting in auto- and paracrine manner, it exerts multiple effects on chondrocytes that promote articular cartilage degradation. These include inhibition of ECM synthesis, activation of MMPs and other matrix-degrading enzymes, increased susceptibility to injury by other oxidants (e.g., H_2O_2) and apoptosis. (Di Cesare et al., 2021) A downstream mediator of proinflammatory cytokine -induced cartilage damage that has been increasingly recognized in recent years is HIF2α. Like HIF1α (see above), it is physiologically induced by low oxygen levels, but in contrast to the latter's benign effects on cartilage, HIF2α directly induces the expression of several catabolic proteinases such as MMPs 1, 3, 9 and 12 as well as ADAMTS-4 and also indirectly induces ADAMTS-5. Also, HIF2α has been shown to inhibit the protective process of autophagy and promote chondrocyte apoptosis (Di Cesare et al., 2021).

Interestingly, OA chondrocytes have been observed to bear resemblance to classically activated macrophages (see p. 27) due to both cell types, among other things, producing inflammatory mediators such as IL-1β and TNFα in an NF-κB-driven manner (Minguzzi et al., 2018).

Counteracting the aforementioned proinflammatory and degradative changes in chondrocyte phenotype observed in OA are several anti-inflammatory and reparative ones. These processes are driven and regulated by growth factors and other extracellular signaling molecules. Insulin-like growth factor (IGF-1) is a major factor promoting chondrocyte differentiation and survival, and it also increases ECM component production while opposing procatabolic signals. In OA, the responsiveness of chondrocytes to IGF-1 seems to be disrupted even though IGF-1 levels appear normal or even increased. This disruption has been postulated to be due to increasing presence of ROS in chondrocytes, which alters IGF-1-triggered signaling cascades. The FGF family, especially FGF2 (also known as basic fibroblast growth factor or bFGF), has also been studied extensively in OA. FGF2

is stored in the cartilage matrix from which it is released by mechanical injury or loading. It strongly promotes proliferation in adult chondrocytes, but its effects on ECM production are more unclear with both positive and negative effects having been reported. This is probably due to the differential effects of the FGF receptors (FGFRs) utilized by FGF2: FGFR3 activation appears to be chondroprotective and FGFR1 activation catabolic. Interestingly, FGFR3/FGFR1 ratio has been shown to be reduced in OA, predisposing cartilage to the catabolic effects of FGFR1 activation. FGF18 is another member of the FGF family that has attracted interest in regard to OA pathophysiology and treatment. FGF18 signals through FGFR3 and has anabolic effects on cartilage. (Mobasheri et al., 2021). A recombinant form of this protein (sprifermin) is currently under investigation as a potential disease-modifying drug in OA (Onuora, 2021).

An especially important facilitator of anabolic processes in the cartilage is TGFβ/BMP signaling. This superfamily includes three different TGFβ isoforms, at least 13 different BMP proteins as well as other factors of the activin, inhibin and other subfamilies. TGF-β isoforms are central mediators of chondrocyte differentiation. In mature cartilage, they have several anti-inflammatory and anticatabolic effects: TGF-β1 downregulates the expression of MMP-1 and MMP-13 as well as IL-1β and TNF receptors in OA chondrocytes, while TGF-β2 suppresses the degradation of type II collagen by limiting the expression of MMPs and proinflammatory cytokines. BMPs also stimulate chondrocyte differentiation and ECM production (Mobasheri et al., 2021). A member of the BMP family that has recently got increased attention is growth differentiation factor 5 (GDF5, also known as BMP-14). This protein regulates skeletal development and chondrocyte differentiation, and polymorphisms in the *GDF5* gene have been linked to OA as well as to autosomal recessive chondrodysplasia syndromes. Recombinant GDF5 and other interventions aimed at increasing GDF5 signaling in the cartilage are under investigation as potential therapeutic strategies in OA (Sun et al., 2021).

2.5 Fibrosing diseases

The term "fibrosis" refers to accelerated production of connective tissue in response to an offending insult. In its benign, essential form, it allows tissue to repair following an injury. However, this process can also be activated needlessly and/or in a dysregulated manner, leading to development of a fibrosing disease. These diseases

are characterized by excessive and abnormal deposition of ECM, obliterating normal tissue architecture and disrupting function. (Birbrair et al., 2014; Neary et al., 2015).

Despite displaying a different pattern of leukocyte activation compared to more "traditional" inflammatory diseases such as RA, fibrosing diseases are currently thought to possess central inflammatory components. This is fitting, as inflammation is an unavoidable part of any tissue repair process after birth (Lee & Kalluri, 2010; White & Mantovani, 2013). "Local" fibrosing diseases can affect a variety of organs, most commonly lungs, liver and kidney. Systemic sclerosis (SSc), also known as systemic scleroderma or diffuse scleroderma, causes accumulation of collagen in the skin and visceral organs, as well as vasculopathy (damage to the small arteries). Bleomycin-induced dermal fibrosis in the mouse is widely used as a model of scleroderma, and has been shown to reproduce many features of the disease, such as increased synthesis of collagen and proinflammatory mediators (Błyszczuk et al., 2019; Lei et al., 2016).

Macrophages are thought to initiate fibrosis by secreting profibrotic mediators such as TGFβ, platelet derived growth factor (PDGF), connective tissue growth factor (CTGF) and IL-4. These factors activate fibroblasts, for example via Akt/mTOR (Mitra et al., 2015) and SMAD (Frangogiannis, 2020) pathways, leading to increased production of collagens and glycosaminoglycans. Increased vascular permeability allows macrophages and lymphocytes to invade tissues, and a subsequent profibrotic reaction results in increased collagen deposition. Over time, angiogenesis (formation of new blood vessels) is disrupted, and the intimae of blood vessels thicken. This subjects tissues to hypoxia (Liakouli et al., 2018). T_H2 lymphocyte activation has been shown to play an important role in the development of fibrosis, and levels of IL-4 and IL-13 are increased in patients. Macrophages present in fibrosing disease generally display features of alternative activation (see p. 31) (Morell et al., 2017), and blocking alternative activation with the phosphodiesterase 4 (PDE4) inhibitor rolipram (Maier et al., 2017) and the tyrosine kinase inhibitor nintedanib (Huang et al., 2017) have both been found to ameliorate disease in a murine dermal fibrosis model.

At the intersection of fibrosing diseases and arthritides is the phenomenon of arthrofibrosis, in which there is accumulation of fibrotic scar tissue in a joint and/or the surrounding tissue. This results in painful restriction of movement. Arthrofibrosis may develop as a complication of an injury or surgery, or it can occur

without a specific cause. Adhesive capsulitis of the shoulder ("frozen shoulder") is probably the most well-known example of idiopathic arthrofibrosis. The pathophysiology of arthrofibrosis, and its relation to other fibrosing diseases, is incompletely understood. However, it seems to involve a type of proinflammatory signaling that causes synovial fibroblasts to assume a myofibroblast-like phenotype that produces fibrotic mediators and ECM components. (Usher et al., 2019)

Compared to other rheumatic diseases such as RA, prognosis of which has improved greatly during the previous decades, fibrosing diseases continue to be associated with large morbidity and mortality. Disease-modifying agents have proven difficult to find. Pirfenidone and nintedanib have a modest effect on the progression of pulmonary fibrosis (Raghu et al., 2015), and the latter has recently been approved for the treatment of interstitial lung disease associated with systemic sclerosis, but no disease-modifying treatments have been approved for scleroderma (Campochiaro & Allanore, 2021). Clearly, there is a need for treatment targeting the underlying factors, rather than just the symptoms, of fibrosing diseases.

2.6 MAP kinases and MKP-1

Mitogen-activated protein kinases (MAPKs) regulate a wide array of cellular functions, including proliferation, metabolism, stress responses and inflammation (Korhonen & Moilanen 2014). They are activated by MAP kinase kinases (MKKs) by phosphorylation of (both) tyrosine and threonine residues in their conserved Thr-Xaa-Tyr domains (where Xaa is an amino acid residue characteristic of a distinct MAPK subfamily). MKKs are itself activated by MAPK kinase kinases (MKKKs), forming a multilayered signaling cascade (see Figure 2-3) (Plotnikov et al., 2011). Once activated, MAPKs can phosphorylate a range of other proteins, affecting gene expression with various downstream mechanisms. These include modulation of chromatin structure (Cheung et al., 2000) and enhancement of the activity of transcription factors such as AP-1 (Whitmarsh & Davis, 1996), CREB (Simon et al., 2004), C/EBPβ (Davis, 1993) and SRF (Posern & Treisman, 2006). They can also alter stability, transport, and translation of messenger RNA (mRNA) (Tiedje et al., 2014).

MAPKs are inactivated by desphosphorylation. In mammalian cells, MAPK phosphatases (MKPs), also known as dual specificity phosphatases (DUSPs, so

called because they can dephosphorylate both tyrosine and threonine residues of MAPKs) are thought to be the most important regulators of MAPK function. At least ten distinct MKPs have been identified in mammalian cells. (Seternes et al., 2019)

MKP-1 (DUSP1) is an archetypal MAP kinase phosphatase. Of the various MAPKs, its preferred targets are p38 and JNK kinases, even though it is also capable of dephosphorylating extracellular signal-regulated kinases (ERKs) (Comalada, Lloberas, et al., 2012). As these MAP kinases are central regulators of cellular stress responses and inflammation, it is not surprising that MKP-1 has been identified as a potent endogenous anti-inflammatory factor (Korhonen & Moilanen, 2014). Macrophages from MKP-1 deficient mice produce greater amounts of proinflammatory factors such as TNFα, IL-6, and iNOS (Korhonen et al., 2011; Turpeinen et al., 2011). In contrast, the effects of MKP-1, if any, on the anti-inflammatory alternative macrophage activation (see p. 31) are largely unknown. *In vivo*, MKP-1 deficient mice display stronger and longer-lasting inflammatory responses. Compared to wild-type mice, they are predisposed to more severe disease in the collagen-induced arthritis model (Sartori et al., 2009; Vattakuzhi et al., 2012) and to increased lethality in sepsis and endotoxin shock (Abraham et al., 2006; Frazier et al., 2009). MKP-1 has also been shown to mediate the effects of various anti-inflammatory medications such as the gold compound aurothiomalate, the PDE4 inhibitor rolipram, the beta2-receptor agonist salbutamol and the glucocorticoid dexamethasone (Keränen et al., 2016; Korhonen et al., 2013; Nieminen et al., 2010; Tuure et al., 2017)

Many extracellular stimuli including stress, growth factors, proinflammatory cytokines and bacterial toxins, activate MKP-1 transcription. MAPKs also promote its transcription, forming a negative feedback loop that is thought to act as an endogenous "brake" for excessive, harmful inflammation (Keyse, 2000; Seternes et al., 2019). In macrophages challenged by LPS, this seems to be mediated by both p38 and JNK kinases, as well as ERK (Ananieva et al., 2008; Chen et al., 2002). The transcription factor NF-κB also seems to mediate MKP-1 transcription induced by TLR activation (Wang, J. et al., 2010). Histone modification, in the form of phosphorylation and acetylation of H3, is implicated in stress-induced MKP-1 transcription (Li et al., 2001). In addition, the microRNA (miRNA) miR-101 mediates MKP-1 translation by binding to the 3' UTR (untranslated region) of MKP-1 mRNA (Zhu et al., 2010). This miRNA is induced by TLR ligands, and therefore

probably acts as a part of an inflammation-limiting feedback loop (Wancket et al., 2012).

Degradation of MKP-1 is mediated by the ubiquitin-directed proteasome complex (Brondello et al., 1999). In LPS-stimulated macrophages, ERK appears to phosphorylate MKP-1, preventing its degradation in the proteasome (Chen et al., 2002). Since p38 and JNK, rather than ERK itself, are the preferred substrates of MKP-1, this is an example of "crosstalk" between the different MAPK pathways.

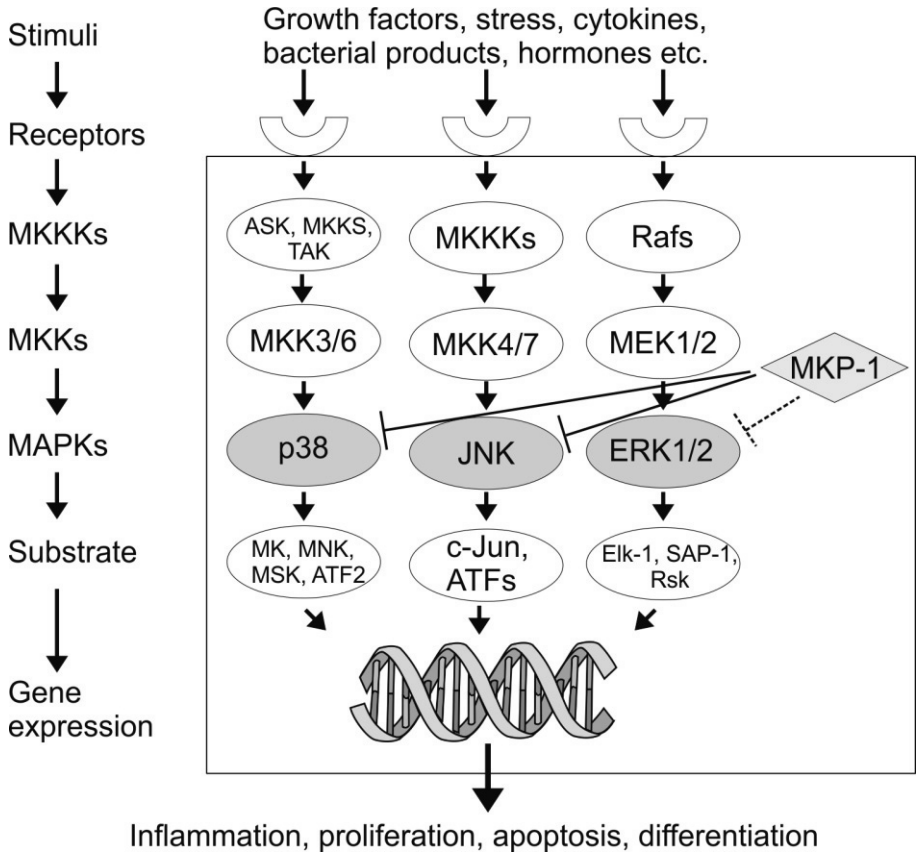

Figure 2-3. An overview of the MAPK pathways and the action of MKP-1. LPS = lipopolysaccharide, ASK = apoptosis signal-regulating kinase, TAK =TGF-beta-activated kinase, MEK = MAP/ERK kinase, JNK = c-Jun N-terminal kinase, ERK = extracellular signal regulated kinase, MKKK = mitogen-activated protein kinase kinase kinase, MKK = mitogen-activated protein kinase kinase, MAPK = mitogen-activated protein kinase, MK = MAPK-activated protein kinase, MNK = MAPK-interacting kinase, MSK = mitogen- and stress-activated kinase, ATF = activated transcription factor, SAP = SRF accessory protein, RSK = ribosomal s6 kinase. Modified from Plotnikov et al. 2011.

2.7 Glucocorticoids

Glucocorticoids are steroid hormones that are produced in the adrenal cortex from cholesterol. They have significant effects on gene expression in a wide variety of tissues, especially affecting glucose and lipid metabolism as well as inflammatory responses. In the setting of inflammation, they reduce the production of proinflammatory effector and signaling molecules and promote the expression of anti-inflammatory factors.

Glucocorticoids are lipophilic molecules that readily penetrate the cell membrane and form a complex with the glucocorticoid receptor (GR) in the cytosol. GR then dimerizes and migrates into the nucleus. The GR-steroid complex binds to glucocorticoid response elements (GREs) in the promoter region of target genes, promoting their transcription. They can also inhibit the expression of genes via a process called transrepression (Barnes, 2016). This can involve a direct protein-protein interaction between the GR-steroid complex and a transcription factor. The GR-steroid complex can also inhibit the effect of a transcription factor that has already attached to DNA. GCs may also reduce transcription indirectly by increasing the expression of factors that inhibit transcription factors, such as the NF-κB-counteracting inhibitor of nuclear factor κB (IκB). Further, GCs act via microRNAs to enchance the degradation of mRNAs coding several proinflammatory mediators. Induction of the anti-inflammatory phosphatase MKP-1, which inhibits MAPK signaling (see p. 52) is an another important anti-inflammatory effect of GCs (Tuure et al., 2017). As the effects of GCs are primarily mediated via altered gene expression, they are relatively slow, taking from several hours to over a day to fully manifest. (Leppänen & Moilanen, 2018) However, significantly faster "nongenomic" effects have also been described. The mechanisms of these effects are still unclear, but theories involving GCs interacting with cell membranes, interaction with G-protein-coupled receptors, as well signaling via the relatively understudied membrane-bound glucocorticoid receptors (mGRs) have been proposed (Savvidou et al., 2019).

As medications, GCs are most often used for their anti-inflammatory effects. These effects are mediated both via induction of anti-inflammatory factors and by suppression of proinflammatory factors through the inhibition of transcription factors, most famously NF-κB and activator protein 1 (AP-1) or their cofactors (Barnes, 2016). GCs decrease the synthesis of proinflammatory prostaglandins (PGs) and leukotrienes by promoting the transcription of annexin-1 which inhibits

phospholipase A_2, the first step in the eicosanoid pathway. In addition, GCs reduce inflammation and pain via decreasing the production of PGE_2 via reduced expression of *COX2* and microsomal prostaglandin E synthase (*PTGES*). They also reduce the production of bradykinin and NGF (Leppänen & Moilanen, 2018). They also reduce the expression of transient receptor potential ankyrin 1 (TRPA1), an ion channel implicated to play a role in cartilage degradation and pain in arthritis (Nummenmaa et al., 2017).

GCs have profound effects on metabolism in several cell types. As stress hormones, they generally act to mobilize energy reserves into readily useful forms, promoting gluconeogenesis (production of glucose from amino acids) and increasing resistance to insulin (which acts to store energy). GCs also increase protein degradation and lipolysis (mobilization of fatty acids from storage) (Ritter et al., 2018b). Their effects on metabolism in chondrocytes, cells characterized by relatively unique hypoxia-adapted and glycolysis-dominated energy metabolism, seem to be much less studied (Black & Grodzinsky, 2019).

Intra-articular glucocorticoids are widely used in the treatment of OA exacerbations, but their net effects on cartilage are largely unknown. Laboratory studies have identified a large number of both potentially beneficial and detrimental effects; the balance between these may depend on factors such as GC concentration and duration of action as well as the phenotype and phase of OA. It has been postulated that modest doses over short periods of time produce beneficial effects, while high doses over long periods may be detrimental (Wernecke et al., 2015). There is evidence that GCs can promote the production of ECM components and reduce the production of degradative enzymes such as MMPs. However, some *in vitro* studies have shown GCs to reduce chondrocyte viability by increasing oxidative stress and inducing apoptosis (Suntiparpluacha et al., 2016) while inducing factors such as lipocalin 2 (LCN2) that impairs chondrocyte phenotype and function. Also, the expression of chondroprotective factors such as TIMP3 may be reduced by GCs. (Savvidou et al., 2019) Another avenue by which they may affect chondrocyte health is via their metabolic effects (see above). Osteoarthritis is linked to metabolic disturbances, and there is evidence of metabolic alterations in OA chondrocytes; impairments in glycolysis (Ruiz-Romero et al., 2008), cholesterol metabolism (Choi et al., 2019) and mitochondrial respiration (Lane et al., 2015) have been reported. Thus, GCs could plausibly influence OA pathogenesis especially in the context of the so-called metabolic phenotype of OA.

Some studies have suggested that repeated GC injections may accelerate cartilage degradation, cause joint space narrowing and lower cartilage volume. X-ray studies have generally found no evidence of joint space narrowing (Raynauld et al., 2003; Spitzer et al., 2019), while some MRI studies have shown repeated injections to reduce cartilage volume (McAlindon et al., 2017). Also, there is some evidence that GC injections may reduce the levels of cartilage turnover markers in serum and urine, suggesting that they reduce cartilage degradation (Klocke et al., 2018). Thus, the risk/benefit ratio of intra-articular GCs is unclear, and their detailed effects on chondrocyte phenotype is especially understudied. Interestingly, GR expression has been shown to be decreased in chondrocytes from OA patients compared with healthy cells (DiBattista et al., 1993); the relevance and implications of this finding remain unclear (Macfarlane et al., 2020).

2.8 Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a group of compounds widely used for pain (especially musculoskeletal pain, e.g. different forms of arthritis) and fever (Chen & Dragoo, 2013). They work by inhibiting the synthesis of prostanoids by the COX enzymes. COX-1 is the "tonic" producer of eicosanoids, active in physiological conditions, while COX-2 is strongly induced in inflammation. The substrate for these enzymes is arachidonic acid, released from the cellular membranes via the action of phospholipase A_2 (PLA2). Arachidonic acid is then converted into the endoperoxides PGG_2 and PGH_2, which are in turn converted by specific synthases to prostanoids such as prostacyclin (PGI_2), prostaglandins D_2, E_2 and $F_{2\alpha}$ and thromboxane (TXA_2). In inflammation, PGE_2 acts as an important mediator of fever and pain. Nonselective NSAIDs inhibit both COX-1 and COX-2 isoforms, while COX-2-selective compounds (e.g. coxibs) inhibit only COX-2 at clinically used doses. As stated before, inhibition of prostanoid synthesis effectively reduces inflammation-associated pain and dampens some other aspects of inflammation. However, despite their name, their direct anti-inflammatory effects are quite modest compared to e.g. glucocorticoids. Thus, they possess significantly less "disease modifying" activity in inflammatory diseases, mainly acting as symptomatic treatments. (Ritter et al., 2018a)

Adverse effects of NSAIDs include increased risk of gastrointestinal bleeding caused by the inhibition of PGE_2 production which is important for the maintenance of

healthy stomach/duodenal wall. Kidney function is also impeded due to reduced blood flow into the organ. NSAIDs have also been linked to increased risk of cardiovascular events. This is probably due to several factors: altered balance between vasodilatory, antithrombotic prostacyclin and vasoconstrictive, prothrombotic thromboxane; increased blood pressure; changes in water and sodium excretion; and impaired endothelial NO synthesis. COX-2 selective agents have greatly reduced risk of gastrointestinal adverse events but unfortunately share the risks of kidney damage and increased cardiovascular events; indeed, the risk of myocardial infarction and stroke tend to be higher for coxibs than many nonselective NSAIDs. Despite these potential adverse effects, NSAIDs have low acute toxicity and are generally well-tolerated in short to medium-term use by patients lacking other risk factors. (Ritter et al., 2018a)

As NSAIDs have a relatively favorable side effect profile and are widely available over the counter, they are a commonly used in symptomatic treatment of musculoskeletal pain, including in OA. However, their potential effects on cartilage are somewhat controversial. As chronic low-grade inflammation is a central feature of OA, and PGE_2 in particular has been linked to cartilage degradation *in vitro* (Di Cesare et al., 2021; Hardy et al., 2002), NSAIDs have been hypothesized to (modestly) slow down OA progression (Nakata et al., 2018). Accordingly, *in vitro*, NSAIDs have been shown to inhibit chondrocyte apoptosis and dedifferentiation as well as to reduce the production of NO and catabolic MMPs (Williams et al., 2013). Also, the NSAID ibuprofen has been reported to decrease markers of joint tissue turnover in knee OA (Gineyts et al., 2004). Interestingly, some of the anti-inflammatory and anticatabolic effects of NSAIDs may be independent of COX inhibition, possibly mediated instead via modulation of p38 or ERK MAP kinases, or by protein kinase C α and ζ subtypes (Z. Li et al., 2015; Yoon et al., 2003).

Despite the putative beneficial effects of NSAIDs on chondrocytes, potentially deleterious effects have also been reported. *In vitro*, NSAIDs may inhibit the anabolic functions of chondrocytes, and observational studies have suggested that they can accelerate radiographic OA progression. However, reported *in vitro* results vary substantially between different NSAIDs and experimental conditions (Ding, 2002). Radiographic studies, in turn, have been criticized for, among other things, not controlling for the severity of joint pain as a potential confounder (Ding, 2006). To date, evidence concerning the net effects of NSAIDs on cartilage appears

inconclusive (Nakata et al., 2018), and their detailed effects on chondrocyte phenotype are unknown.

2.9 Plant polyphenols

Polyphenols are a group of organic compounds containing several phenol units. They are synthesized by various plants, in which they perform several roles, including acting as antioxidants, antibacterial agents (phytoalexins), UV screens and signaling molecules (Huber et al., 2003; Lattanzio et al., 2006). Plant extracts rich in these compounds are traditionally used in folk medicine (Shay et al., 2015). In recent decades, they have also attracted considerable interest due to their potential beneficial biological effects in mammals, especially in regards to their antioxidant and anti-inflammatory activities (Quideau et al., 2011). Compounds of the stilbenoid and lignan classes are well-known examples of bioactive polyphenols; they have been investigated as potential novel drug candidates from natural products (Patel, 2017). An example of a natural product rich in these compounds is the extract obtained from the knots (parts of the branch residing inside the tree) of Scots pine (*pinus sylvestris*).

Stilbenoids are hydroxylated derivatives of stilbene, a compound synthesized from the amino acid phenylalanine. Many of them have been shown to possess antibacterial and antifungal properties (Välimaa et al., 2007). Resveratrol, the major stilbenoid in grapes and red wine, has attracted much attention due to its potential health benefits, though robust evidence of any benefits in human is currently lacking (Akinwumi et al., 2018). The stilbenoids pinosylvin and monomethyl pinosylvin have previously been shown to strongly suppress classical macrophage activation in an NF-κB mediated manner (Lee et al., 2006), decrease the production of inflammatory mediators in macrophages (Eräsalo et al., 2018; Laavola et al., 2015) and to suppress carrageenan-induced paw edema in a murine model (Laavola et al., 2015). They have also been shown to promote M2 macrophage activation in a PPARγ-mediated manner (Kivimäki et al., 2021). In chondrocytes, stilbenoids increase the expression of the cartilage ECM component aggrecan and to reduce IL-6 expression (Laavola et al., 2019).

Lignans are also derived from phenylalanine, via dimerization of substituted cinnamic alcohols to a dibenzylbutane skeleton (Heinonen et al., 2001). Many of

them have antioxidative and anti-inflammatory effects, which have been shown to protect animals in models of experimental cerebral ischemia-reperfusion injury (Baumgartner et al., 2011; Zhang et al., 2013), carrageenan-induced paw inflammation (Laavola et al., 2017) and sepsis (Li et al., 2014). These effects appear to be at least partly mediated by attenuation of proinflammatory "classical" macrophage activation (Laavola et al., 2017). However, as far as we are aware, the potential effects of lignans on alternative macrophage activation have remained largely unexplored.

3 AIMS OF THE STUDY

Analogously to T helper (T_H) cells, macrophages have been shown to adopt distinct phenotypes in response to environmental cues and phase of the inflammatory response. Changes in these phenotypes have been linked to diseases. As macrophages are central regulators of inflammatory response, modulating macrophage phenotype is a compelling potential approach to alleviating inflammatory and fibrotic diseases. Phenotypic changes in chondrocytes have been observed in arthritis, and OA chondrocytes have been noted to bear resemblance to proinflammatory "classically activated" macrophages (Minguzzi et al., 2018). Thus, an intriguing question is whether chondrocytes can adopt different phenotypes analogous to polarized macrophages. Investigating the endogenous and exogenous factors affecting these phenotypes may open avenues for understanding their biology and pathophysiology, and eventually for pharmacologically modulating them. This might, in turn, offer potential new treatments for inflammatory diseases.

The detailed aims of the present study were:

I) To investigate the potential role of the phosphatase MKP-1 in anti-inflammatory and tissue-healing alternative macrophage activation.

II) To study the effects of Scots pine (*Pinus sylvestris*) knot extract and its lignan compound nortrachelogenin on alternative macrophage activation and fibrosis.

III) To characterize the chondrocyte phenotypes induced by the central $T_H1/T_H2/T_H17$ cytokines in OA chondrocytes.

IV) To investigate the effects of the glucocorticoid dexamethasone on the phenotype of chondrocytes from OA patients.

V) To study the effects of the NSAID ibuprofen on OA chondrocyte phenotype.

4 MATERIALS AND METHODS

4.1 Materials

Table 4-1. Chemicals, antibodies and ELISA kits used in the study

Chemicals	**Supplier**
Interleukin 4	R&D Systems Europe Ltd., Abingdon, UK
Interleukin 13	R&D Systems Europe Ltd., Abingdon, UK
Nortrachelogenin	Arbonova, Turku, Finland
Matairesinol	Arbonova, Turku, Finland
Pinoresinol	Arbonova, Turku, Finland
Secoisolariciresinol	Arbonova, Turku, Finland
Conidendrin	Arbonova, Turku, Finland
Pinosylvin	Sequoia Research Products, Pangbourne, UK
Monomethyl pinosylvin	Sequoia Research Products, Pangbourne, UK
Abietic acid	Alfa Aesar, Haverhill, MA, USA
Pimaric acid	MP Biomedicals, Santa Ana, CA, USA
Hydroxymatairesinol	Sigma-Aldrich, St. Louis, MO, USA
Bleomycin	Cayman Chemical, Ann Arbor, MI, USA
E. Coli LPS (product no. E-4391)	Sigma-Aldrich, St. Louis, MO, USA
Ibuprofen	Sigma-Aldrich, St. Louis, MO, USA
Dexamethasone	Sigma-Aldrich, St. Louis, MO, USA
Antibodies	
Rabbit polyclonal β-actin (sc-1615R)	Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA
Rabbit polyclonal arginase 1 (sc-18351)	Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA
Rabbit polyclonal lamin A/C (sc-20681)	Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA
Rabbit polyclonal STAT6 (sc-981)	Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA
Rabbit polyclonal pSTAT6 (ab54461)	Abcam, Cambridge, UK
ELISA kits	
Murine IL- 6 (catalog no. DY406)	R&D Systems Europe Ltd., Abingdon, UK
Murine TNFα (catalog no. DY410)	R&D Systems Europe Ltd., Abingdon, UK
Human CCL13 (catalog no. DY327)	R&D Systems Europe Ltd., Abingdon, UK
Human MMP-1 (catalog no. DY901)	R&D Systems Europe Ltd., Abingdon, UK
Human MMP-13 (catalog no. DY511)	R&D Systems Europe Ltd., Abingdon, UK
Human CCL2/MCP-1 (catalog no. DY279)	R&D Systems Europe Ltd., Abingdon, UK

All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

4.1.1 *Pinus sylvestris* knot extract

In Study II, Scots pine (*Pinus sylvestris*) knot extract was prepared as described previously (Laavola et al., 2015). In brief, dry knot material was ground, extracted using an accelerated solvent extractor apparatus, and analyzed by gas chromatography-mass spectrometry after silylation. The extract was found to contain the following identified components, as wt % of dry knot material: 12% monomethyl pinosylvin, 12% resin acids (mainly pimaric acid and abietic acid), 11% oxidized resin acids, 7% nortrachelogenin, 5% pinosylvin and 2% matairesinol (Laavola et al., 2015).

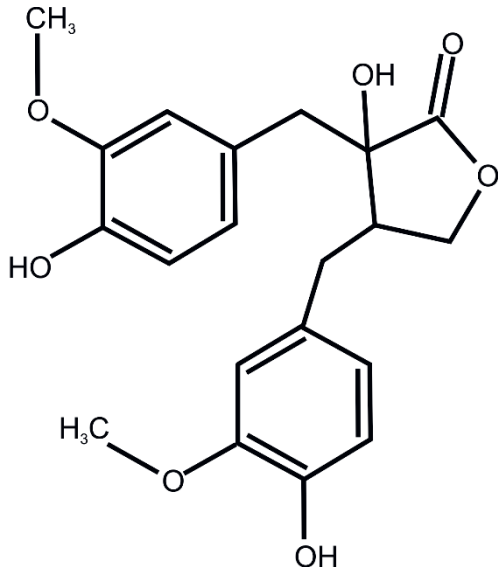

Figure 4-1. Chemical structure of nortrachelogenin.

4.2 Cell isolation and culture

4.2.1 Cell lines

In Studies I and II, murine J774 macrophages [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured at 37°C in a 5% CO_2 atmosphere in DMEM with Ultraglutamine 1 D5796 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin

and 250 ng/ml amphotericin B (Invitrogen Co., Carlsbad, CA, USA) and harvested with trypsin-EDTA (Invitrogen Co.). Cells were cultured in 24-well plates for RNA extraction, ELISA measurements, and preparation of whole-cell lysates for Western blotting. Confluent cultures were exposed to fresh culture medium containing the compounds of interest. In order to induce classical (M1) activation in the cells, they were incubated with 10 ng/ml of bacterial LPS. In experiments examining alternative (M2) activation, a combination of IL-4 and IL-13 (both 10 ng/ml) was used instead. Unless indicated otherwise, the incubation time was 24 hours.

In Study II, THP-1 human monocytes (ATCC) were cultured according to a protocol similar to J774 cells, except that the medium used was RPMI 1640 containing 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate and supplemented with 10% heat-inactivated fetal bovine serum (all from Lonza, Basel, Switzerland), 100 U/ml penicillin,100 μg/ml streptomycin, 250 ng/ml amphotericin B (all from Invitrogen), and 0.05 mM 2-mercaptoethanol. Cells were seeded on a 24-well plate and differentiated into macrophages by adding 100 nM phorbol ester 12-O-tetradecanoylphorbol-13-acetate 72 h before the experiments were started. Confluent cultures were then exposed to fresh culture medium containing the compounds of interest.

Cell viability was investigated using a modified XTT method, measuring mitochondrial dehydrogenase activity in cells via their ability to reduce a tetrazolium salt (Roehm et al., 1991). Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN, USA) was used. Cells were incubated with the tested compounds for 20 h before addition of sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate (final concentration 0.3 mg/ml) and N-methyl dibenzopyrazine methyl sulfate (final concentration 1.25 mM). Cells were then incubated for another three hours, and the amount of formazan secreted into the culture medium was determined spectrophotometrically. Triton X-100 was used as a positive control for cell death. IL-4, IL-13, LPS, *Pinus sylvestris* knot extract or its components (alone or in relevant combinations) were not found to evoke cytotoxicity in either J774 or THP-1 cells in concentrations used in Study II.

4.2.2 Primary murine peritoneal macrophages

Isolation and culturing of mouse peritoneal macrophages (PMs) (used in Study I) were carried out as described earlier (Korhonen et al., 2013). In brief, inbred male C57BL/6 MKP-1(–/–) mice [originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute (Dorfman et al., 1996) and received from Professor Andy Clark, University of Birmingham, UK] were bred in the animal facilities of University of Tampere School of Medicine animal facilities. These mice, along with their wild-type (WT) controls, were housed under conditions of optimum light (12-12 h light–dark cycle), temperature (22 ± 1°C) and humidity (50–60%). Food and water were provided *ad libitum*.

In collection of PMs, the experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by The National Animal Experiment Board. Mice were sacrificed by suffocation with CO_2, followed by an immediate cervical dislocation. Primary mouse PMs were obtained by intraperitoneal lavage, using sterile phosphate-buffered saline (PBS) supplemented with 0.2 mM EDTA. Cells were washed, resuspended in RPMI 1640 medium supplemented with 2% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B and seeded on 24-well plates (3×10^5 cells per well). The cells were incubated overnight and washed with medium to remove non-adherent cells. Thereafter the cells were incubated with either 100 ng/ml of LPS for 4 hours, or with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 hours to induce classical (M1) or alternative (M2) phenotype, respectively.

4.2.3 Primary human OA chondrocytes

In Studies III, IV and V, leftover cartilage pieces were collected from patients undergoing total knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. All patients fulfilled the American College of Rheumatology classification criteria for primary OA (Altman et al., 1986). Patients with diabetes mellitus were excluded from the study; this was done to avoid confounding potential observed metabolic effects with those caused by the disease. The study was approved by the Ethics Committee of Tampere University Hospital, Finland (license ETL R09116), and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Chondrocyte isolation and culture was performed as previously described (Koskinen et al., 2011). Articular cartilage was removed aseptically from subchondral bone using a scalpel and cut into small pieces. The pieces were first washed with PBS. After that, they were incubated overnight in the presence of Liberase™ enzyme (Roche Applied Science, Penzberg, Germany, 0.25 mg/mL) diluted in DMEM with glutamax-I containing penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (all three from Invitrogen, Carlsbad, California, USA) at 37°C. The resulting cell suspension was poured through a 70 μm nylon mesh and centrifuged for five minutes at 1500 rpm. The cells were washed and seeded on 24-well plates (0.2 million cells / ml) and incubated for 24 hours. Thereafter, the experiments were started by adding the compounds of interest to the culture media. In Study III, the cells were treated with 10 ng/ml IFNγ, 100 pg/ml IL-1β, 50 ng/ml IL-17 or with 10 ng/ml IL-4. In study IV, dexamethasone at a concentration of 1 μM was used. Study V used ibuprofen at 10 μM, IL-1β at 100 pg/ml or the combination of both.

4.3 Preparation of protein extracts

At predetermined time points, the culture medium of cells was carefully removed and stored at -20°C for later analysis. For preparation of cell lysates for arginase 1 Western blotting, the cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside. After incubation on ice for 15 minutes, the lysates were centrifuged (12,000 g, 4°C, 10 minutes), supernatants were collected and mixed 3:1 with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). The samples were stored at -20°C until analyzed. An aliquot of the supernatant was used to determine protein concentration using the Coomassie blue method (Bradford, 1976). Nuclear extracts for pSTAT6 Western blotting were prepared as previously described (Salonen et al., 2006). Protein contents of the extracts were again measured by the Coomassie blue method.

4.4 Western blotting

Prior to Western blotting, samples were boiled for 10 min. 20 μg of protein per lane was then loaded onto 10% SDS-polyacrylamide gels. The proteins were separated by electrophoresis in a running buffer containing 25 mM Tris, 250 nM glycine and 0.1% SDS, then electrically transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK). The membrane was blocked in TBS/T solution (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat dry milk (for arginase 1 and STAT6) or 5% bovine serum albumin (for pSTAT6 and MKP-1) for 1 h at room temperature, then incubated with the primary antibody in the blocking solution for 1 h at room temperature (for arginase 1) or at 4 °C overnight (for pSTAT6, STAT6 and MKP-1). After that, the membrane was washed with TBS/T, incubated with horseradish peroxidase (HRP)-containing secondary antibody in the blocking solution for 45 min at room temperature and washed again. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrates (Pierce, Rockford, IL, USA) and Image Quant LAS 4000 mini imaging system (GE Healthcare, Little Chalfont, UK). The chemiluminescent signal was quantified with ImageQuant TL 7.0 image analysis software. β-actin was used as a loading control for cytoplasmic proteins and lamin A/C for nuclear pSTAT6/STAT6.

4.5 Enzyme-linked immunosorbent assay (ELISA)

The concentration of murine IL-6 (Studies I and II) and TNFα (Study II) in the culture media of LPS-treated J774 cells, as well as that of human CCL13 in media of IL-4 + IL-13 –treated THP-1 cells, was determined by ELISA according to the manufacturer's instructions. The detection limit was 7.8 pg/ml for IL-6, 15.6 pg/ml for TNFα and 3.8 pg/ml for CCL13.

In Study IV, the concentrations of MMP-1, MMP-13 and CCL2 (MCP-1) were determined by ELISA in the chondrocyte culture media according to the manufacturer's instructions, with detection limits of 39 pg/ml, 31.3 pg/ml and 7.8 pg/ml, respectively.

4.6 Nitrite measurement with Griess method

In Studies I and II, NO production was determined by measuring the accumulation of nitrite (a stable metabolite of NO in the aqueous environment) into the culture medium. The culture medium was collected at predetermined time points, and nitrite was measured by the Griess reaction (Green et al., 1982).

4.7 RNA extraction

In Studies I and II, at predetermined time points, culture medium was removed and total RNA of the cells was extracted using GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich). For cell lines, total RNA (100 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription Kit and random hexamers (Applied Biosystems, Foster City, CA, USA). For murine PM cell and skin samples, Maxima First Strand cDNA Synthesis Kit (Applied Biosystems) was used.

For cultured chondrocytes in Studies III and IV, total RNA was extracted with GenElute™ Mammalian Total RNA Miniprep kit (Sigma), and RNA was treated with DNAse I (Qiagen, Hilden, Germany). In Study V, RNA was extracted using the RNAeasy Mini Kit (Qiagen) with on-column DNase digestion according to the manufacturer's instruction. RNA concentration and integrity of the samples was confirmed with the 2100 Bioanalyzer (Agilent Technologies).

4.8 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Part of the RNA (150 ng / sample) was reverse-transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA obtained from reverse transcription was diluted 1:20 with RNase-free water and subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Primers and probes for murine *ArgI*, *Il6*, *Nos2/iNOS*, *Dusp1/MKP-1*, *Ym-1* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, used as a control gene), as well as human aggrecan (*ACAN*), collagen type II, alpha 1 (*COL2A1*), *COX*-2, *IL-6*,

MKP-1, *MMP*s 1 and 13, prostaglandin E synthase (*PTGES*), *YKL-40* and *GAPDH* (Table 4-2) were designed using Primer Express® Software (Applied Biosystems, Foster City, CA, USA), optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C, and supplied by Metabion (Martinsried, Germany).

Expression of the following murine genes was measured using TaqMan Gene Expression assays (Applied Biosystems): ADAM metallopeptidase with thrombospondin type 1 motif 6 (*Adamts6*, Hs01058097_m1), collagen 1 alpha 1 (*Col1a1*, Mm00801666_g1), *Col1a2* (Mm00483888_m1) and *Col3a1* (Mm01254476_m1), basic fibroblast growth factor (*Fgf2*, Mm00433287_m1), fibronectin 1 (*Fn1*, Mm01256744_m1), *Il13* (Mm00434204_m1) and *PPAR*γ (Mm01184322_m1).

For human genes, the following TaqMan assays were used: human chemokine (C-C motif) ligand 2 (*CCL2*, Hs00234140_m1), *CCL13* (Hs01033504_g1), carbohydrate (chondroitin 4) sulfotransferase 11 (*CHST11*, Hs00905281_s1), colony stimulating factor 2 (*CSF2*, Hs00929873_m1), *COL9A1* (Hs00932136_g1), *COL11A1* (Hs01097664_m1), cysteine rich secretory protein LCCL domain containing 1 (*CRISPLD1*, Hs00230253_m1), filamin A interacting protein 1 like (*FILIP1L*, Hs00706279_s1), forkhead box O3 (*FOXO3*, Hs00818121_m1), hyaluronan synthase 1 (*HAS1*, Hs00758053_m1), insulin-like growth factor-binding protein 4 (*IGFBP4*, Hs01057900_m1), interleukin 23 subunit alpha (*IL23A*, Hs00372324_m1), Krüppel like factor 9 (*KLF9*, Hs00230918_m1), MAP kinase phosphatase 2 (*MKP*-2, Hs01027785_m1), matrix metalloproteinase 16 (*MMP16*, Hs00234676_m1), nerve growth factor (*NGF*, Hs00171458_m1), platelet-derived growth factor beta (*PDGFB*, Hs00966522_m1), *PPAR*γ (Hs01115513_m1), peroxisome proliferator-activated receptor gamma coactivator 1-beta (*PPARGC1B*, Hs00993805_m1), tumor necrosis factor superfamily member 15 (*TNFSF15*, Hs00270802_s1), vascular endothelial growth factor A (*VEGFA*, Hs00900055_m1) and *VEGFC* (Hs01099203_m1).

The PCR reaction parameters were as follows: incubation at 50°C for 2 minutes, incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 minute. Each sample was assayed in duplicate. For self-designed primers and probes, the relative mRNA levels were quantified and compared using the relative standard curve method as described in

Applied Biosystems User Bulletin number 2. For the TaqMan Gene Expression Assays, $2^{(-\Delta\Delta CT)}$ method was used instead. According to the method, the cycle threshold (C_T) value for each gene was normalized to the C_T value of GAPDH mRNA in the same sample.

Table 4-2. Self-designed primers and probes used in RT-qPCR

mArgI forward	5'-TCCAAGCCAAAGTCCTTAGAGATTAT-3'
mArgI reverse	5'-CGTCATACTCTGTTTCTTTAAGTTTTTCC-3'
mArgI probe	5'-Fam-CGCCTTTCTCAAAAGGACAGCCTCGA-TAMRA-3'
mGapdh forward	5'-GCATGGCCTTCCGTGTTC-3'
mGapdh reverse	5'-GATGTCATCATACTTGGCAGGTTT-3'
mGapdh probe	5'-Fam-TCGTGGATCTGACGTGCCGCC-TAMRA-3'
mIl6 forward	5'-TCGGAGGCTTAATTACACATGTTC-3'
mIl6 reverse	5'-CAAGTGCATCATCGTTGTTCATAC-3'
mIl6 probe	5'-Fam-CAGAATTGCCATTGCACAACTCTTTTCTCA-TAMRA-3'
miNos forward	5'-CCTGGTACGGGCATTGCT-3'
miNos reverse	5'-GCTCATGCGGCCTCCTT-3'
miNos probe	5'-Fam-CAGCAGCGGCTCCATGACTCCC-TAMRA-3'
mMkp-1 forward	5'-CTCCTGGTTCAACGAGGCTATT-3'
mMkp-1 reverse	5'-TGCCGGCCTGGCAAT-3'
mMkp-1 probe	5'-Fam-CCATCAAGGATGCTGGAGGGAGAGTGTT-TAMRA-3'
mYm-1 forward	5'-AGTGGGTTGGTTATGACAATGTCA-3'
mYm-1 reverse	5'-GACCACGGCACCTCCTAAATT-3'
mYm-1 probe	5'- Fam-AGCTTCAAGTTGAAGGCTCAGTGGCTCA-TAMRA-3'
hACAN forward	5'-GCCTGCGCTCCAATGACT-3'
hACAN reverse	5'-TAATGGAACACGATGCCTTTCA-3'
hACAN probe	5'- Fam-CCATGCATCACCTCGCAGCGGTA- TAMRA-3'
hCOL2A1 forward	5'-GGCAATAGCAGGTTCACGTACA-3'
hCOL2A1 reverse	5'-CGATAACAGTCTTGCCCCACTT-3'
hCOL2A1 probe	5'- Fam-CTGAAGGATGGCTGCACGAAACATACC- TAMRA-3'
hCOX-2 forward	5'-CAACTCTATATTGCTGGAACATGGA-3'
hCOX-2 reverse	5'-TGGAAGCCTGTGATACTTTCTGTACT-3'
hCOX-2 probe	5'-Fam-TCCTACCACCAGCAACCCTGCCA- TAMRA-3'
hGAPDH forward	5'-AAGGTCGGAGTCAACGGATTT-3'
hGAPDH reverse	5'-GCAACAATATCCACTTTACCAGAGTTAA-3'
hGAPDH probe	5'-CGCCTGGTCACCAGGGCTGC-3'
hIL-6 forward	5'-TACCCCCAGGAGAAGATTCCA-3'
hIL-6 reverse	5'-CCGTCGAGGATGTACCGAATT-3'
hIL-6 probe	5'-Fam-CGCCCCACACAGACAGCCACTC-TAMRA-3'
hMKP-1 forward	5'-ACGAGGCCATTGACTTCATAGAC-3'
hMKP-1 reverse	5'-TCGATTAGTCCTCATAAGGTAAGCAA-3'
hMKP-1 probe	5'-Fam-CCACTGCCAGGCAGGCATTTCC- TAMRA-3'
hMMP1 forward	5'-TTGAAGCTGCTTACGAATTTGC-3'
hMMP1 reverse	5'-GAAGCCAAAGGAGCTGTAGATGTC-3'
hMMP1 probe	5'- Fam-CAGAGATGAAGTCCGGTTTTTCAAAGGGA- TAMRA-3'
hMMP13 forward	5'-TGATCTCTTTTGGAATTAAGGAGCAT-3'
hMMP13 reverse	5'-GGAACTACTTGTCCAGGTTTCATCAT-3'
hMMP13 probe	5'- Fam-CCCTCTGGCCTGCTGGCTCATG- TAMRA-3'
hPTGES forward	5'-CACGCTGCTGGTCATCAAGA-3'
hPTGES reverse	5'-CCGTGTCTCAGGGCATCCT-3'
hPTGES probe	5'-Fam-AGCCTCACTTGGCCCGTGATG- TAMRA-3'
hYKL-40 forward	5'-TGACGCTCTACGGCATGCT-3'
hYKL-40 reverse	5'-TCCATCCTCCGACAGACAAGA-3'
hYKL-40 probe	5'-Fam-AACACACTCAAGAACAGGAACCCCAACCT- TAMRA-3'

4.9 Next generation sequencing (NGS) and data analysis

In Studies III and IV, sequencing of chondrocyte RNA samples was performed in the Finnish Institute of Molecular Medicine (FIMM) sequencing core, Helsinki, Finland, using the Illumina HiSeq 2500 sequencing platform. Sequencing depth was 20 million single-end reads 100 bp in length.

In Study V, RNA samples were sequenced in the Biomedicum Functional Genomics Unit, University of Helsinki, Finland using the Illumina NextSeq 500 system. Sequencing depth was 15 million paired-end reads 100 bp in length.

In both studies, read quality was first assessed using FastQC (Andrews, 2010), and the reads were trimmed using Trimmomatic (Bolger et al., 2014). Trimmed reads were aligned to reference human genome with STAR (Dobin et al., 2013). Count matrices were prepared with the featureCounts program (Liao et al., 2014). Differential expression was assessed with DESeq2 (Love et al., 2014). Functional analysis [using the Gene Ontology (GO) knowledgebase] was performed with the DAVID tool (Huang et al., 2009), and protein interactions were studied with STRING (Szklarczyk et al., 2015). In Study V, REVIGO (Supek et al., 2011) was used to reduce the gene lists obtained from DAVID. Gene functions were obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene). In Study V, Ingenuity Pathway Analysis (IPA) was used to study the functions (activated or inhibited canonical pathways) of the differentially expressed genes (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

4.10 Mouse model of bleomycin-induced dermal fibrosis

Male C57BL/6N mice (Scanbur Research A/S, Karlslunde, Denmark), aged ten weeks, were used to study the effects of nortrachelogenin in bleomycin-induced dermal fibrosis. Mice were bred in the University of Tampere animal facility under standard conditions. The study was approved by the National Animal Experiment Board (ESAVI/10109/04.10.07/2015), and experiments were carried out in compliance with Directive 2010/63/EU.

Bleomycin was dissolved in sterile PBS to give the final concentration of 5 mg/ml. Nortrachelogenin was dissolved in DMSO and diluted in sterile PBS to 20 mg/ml.

The upper dorsa of mice were shaved, and a marker was used to draw a square of about 1.5 cm^2. Mice were anesthetized with sevoflurane inhalation, then injected with either bleomycin (100 µl) or bleomycin (100 µl) and nortrachelogenin (12,5 µl) (eight mice in each group) every other day for 21 days. The injection site in the shaved area was rotated, i.e. first four injections were administered into the corners of the square and the fifth into the middle. On the day after the last injection, the mice were euthanized with CO_2, followed by immediate cervical dislocation. The injected skin (marked square created on the dorsal surface) was cut with a punch. Skin specimens were fixed in 10% formalin for 24 h and processed for histological analyses or stored in RNA*later*™ solution (Invitrogen) and processed for RNA extraction.

In the histological analysis, 6 µm sections of skin were cut, mounted on a slide, and stained with Hematoxylin and Eosin (HE) (Histolab Products AB, Göteborg, Sweden). Dermal thickness (µm) was measured in HE-stained sections by measuring the distance between the epidermal-dermal junction and the dermal-fat junction at six randomly chosen locations in each section using the ImageJ program (National Institutes of Health, Bethesda, MD, USA).

4.11 Statistics

Results (excluding RNA-Seq data) are expressed as mean + standard error of mean (SEM). When indicated, statistical significance was calculated by analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's multiple comparisons test where appropriate. Differences were considered significant at $p < 0.05$. For determining area under the curve, trapezoidal integration was used. Data were analyzed using the Prism computerized package (Graph Pad Software, San Diego, CA, USA).

For RNA-Seq data analysis, normalization was performed and differential expression studied using a negative binomial model implemented in DESeq2.

5 SUMMARY OF THE RESULTS

5.1 Macrophage phenotype is regulated by MKP-1, glucocorticoids and nortrachelogenin

5.1.1 MKP-1 deficiency promotes classical and attenuates alternative macrophage activation (I)

First, we established that the proinflammatory classical (M1) and anti-inflammatory alternative (M2) phenotypes can be induced in J774 murine macrophages. We also determined the optimal time points for studying these phenotypes. Expression levels of interleukin 6 (*Il6*) and inducible nitric oxide synthase (*iNOS*) were measured in J774 cells as markers of classical activation following lipopolysaccharide (LPS) stimulation. LPS enhanced the expression of both of these factors, and mRNA levels peaked at 4-6 h (Figure 5-1, A-B). Arginase 1 and Ym-1 were measured as typical markers of alternative activation. The combination of IL-4 and IL-13 increased the expression of both *ArgI* and *Ym-1*; mRNA levels continued to increase up to 12-24 h (Figure 5-1, C-D).

To study the role of MKP-1 in classical and alternative macrophage activation, peritoneal macrophages were harvested from MKP-1 deficient (knock-out, KO) and wild-type (WT) mice. After stimulation with LPS, expression levels of *Il6* and *iNOS* were higher in macrophages from MKP-1 deficient mice compared to cells from WT mice (Figure 5-2, A-B). When the cells were treated with a combination of IL-4 and IL-13 to induce alternative activation, mRNA levels of *ArgI*, *Ym-1* and *Fgf2* were significantly lower in the macrophages from KO mice than in those from WT animals (Figure 5-2, C-E).

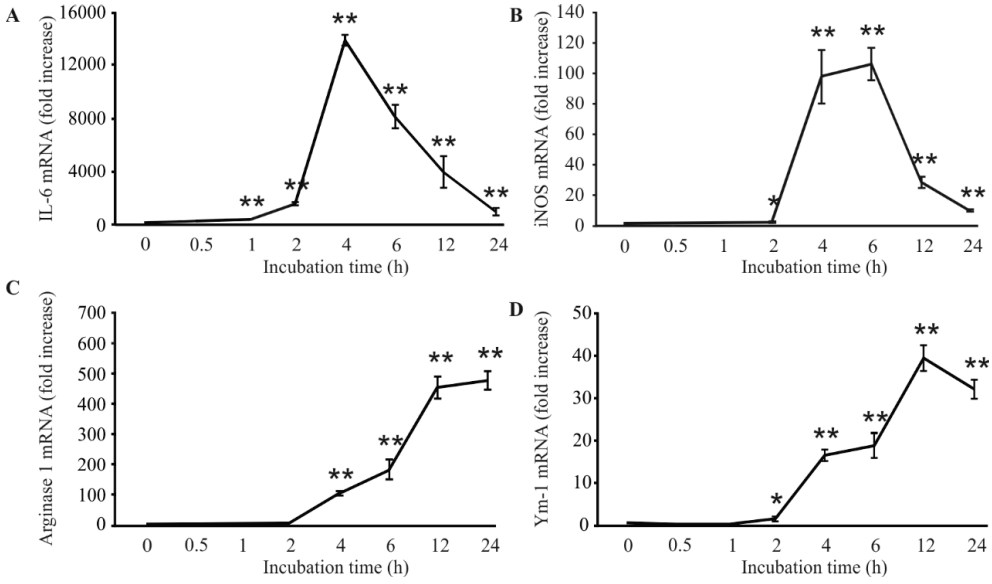

Figure 5-1. LPS and combination of IL-4 and IL-13 enhanced the expression of markers of the classical and alternative macrophage phenotypes, respectively. J774 macrophages were cultured with LPS (10 ng/ml) (A and B) or a combination of IL-4 and IL-13 (both 10 ng/ml) (C-D) for 0.5 – 24 h to induce the respective phenotypes. mRNA levels of the classical activation markers *Il6* (A) and *iNOS* (B), as well as the alternative activation markers *Argl* (C) and *Ym-1* (D) were measured with quantitative RT-PCR and their expression levels were normalized against *Gapdh*. The values were compared to the untreated control (0 h) which was set as 1. The results are expressed as mean ± SEM, n = 4. **: $p < 0.01$ and *: $p < 0.05$, compared to the 0 h time point. Reprinted with permission from Pemmari et al. Basic Clin Pharmacol Toxicol. 2019, 124(4):404-415. Copyright 2018 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

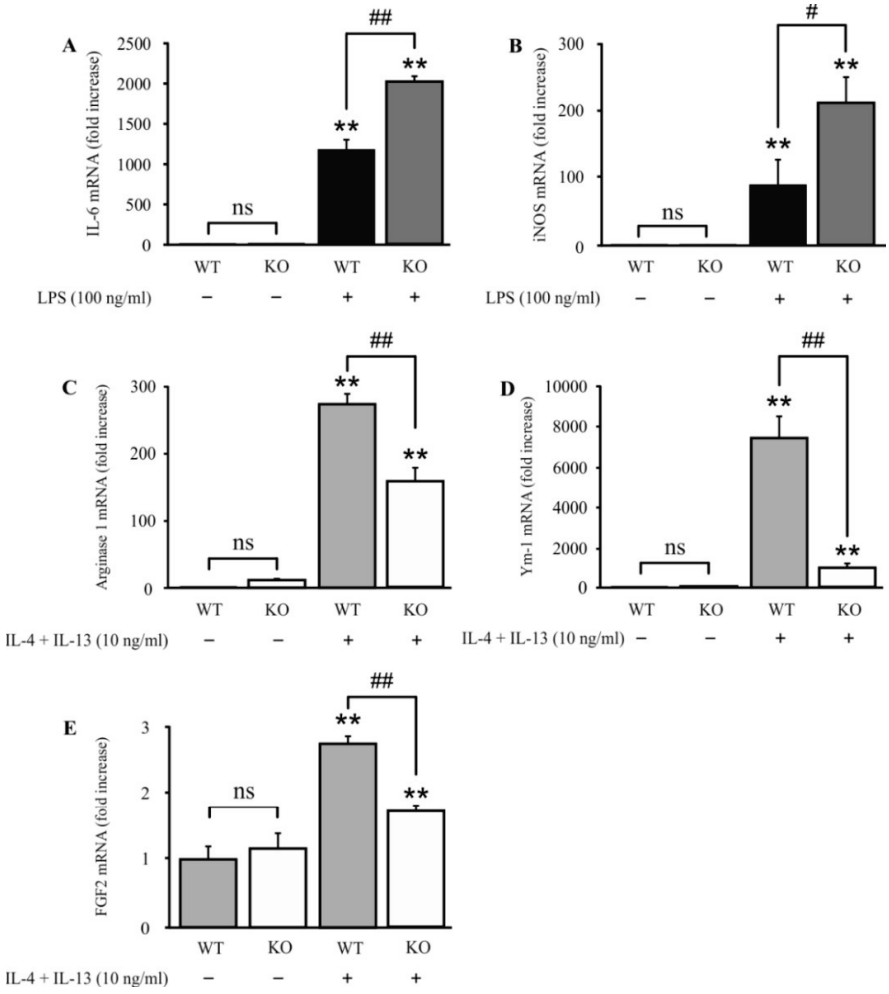

Figure 5-2. MKP-1 deficiency increased the expression of markers of classical phenotype and decreased the expression of markers of alternative phenotype in murine macrophages. Peritoneal macrophages from WT and MKP-1 deficient (KO) mice were cultured with either LPS (100 ng/ml) for 4 h (A-B), or with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 h (C-E). mRNA levels of the classical activation markers *Il6* (A) and *iNOS* (B) as well as the alternative activation markers *Argl* (C), *Ym-1* (D) and *Fgf2* (E) were measured with RT-qPCR and their expression levels were normalized against *Gapdh*. The WT control was set as 1 and the other groups scaled accordingly. The results are expressed as mean + SEM, cells from six WT and six KO mice were pooled together and then divided to give n = 4. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample. The experiments were repeated three times with similar results. Reprinted with permission from Pemmari et al. Basic Clin Pharmacol Toxicol. 2019, 124(4):404-415. Copyright 2018 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

5.1.2 Glucocorticoids suppress classical and promote alternative activation of macrophages, and these effects are partly mediated by MKP-1 (I)

Glucocorticoids have previously been shown to attenuate classical and to promote alternative macrophage activation (Abraham et al., 2006; Martinez et al., 2008). This was also confirmed in the present study in J774 cells. In cells treated with LPS, dexamethasone markedly inhibited *Il6* and *iNOS* mRNA expression as well as IL-6 and NO production (Figure 5-3). Conversely, dexamethasone increased the expression of *ArgI* and *Ym-1* in macrophages exposed to the combination of IL-4 and IL-13 (Figure 5-4).

Interestingly, MKP-1 is known to mediate the attenuating effects of GCs on classical macrophage activation (Abraham et al., 2006). Thus, we set out to study whether the effects of GCs on alternative activation might also be mediated by MKP-1.

Dexamethasone reduced *IL-6* and *iNOS* expression in both WT and MKP-1 deficient peritoneal macrophages exposed to LPS, but the effect was significantly smaller in the latter (Figures 5-5, A-B). Intriguingly, dexamethasone-induced upregulation of alternative activation markers was also significantly attenuated in MKP-1 deficient cells (Figure 5-5, C-E). These observations suggest that MKP-1 mediates the anti-inflammatory effects of GCs via two distinct mechanisms: both by suppressing proinflammatory classical macrophage activation and promoting the anti-inflammatory alternative phenotype.

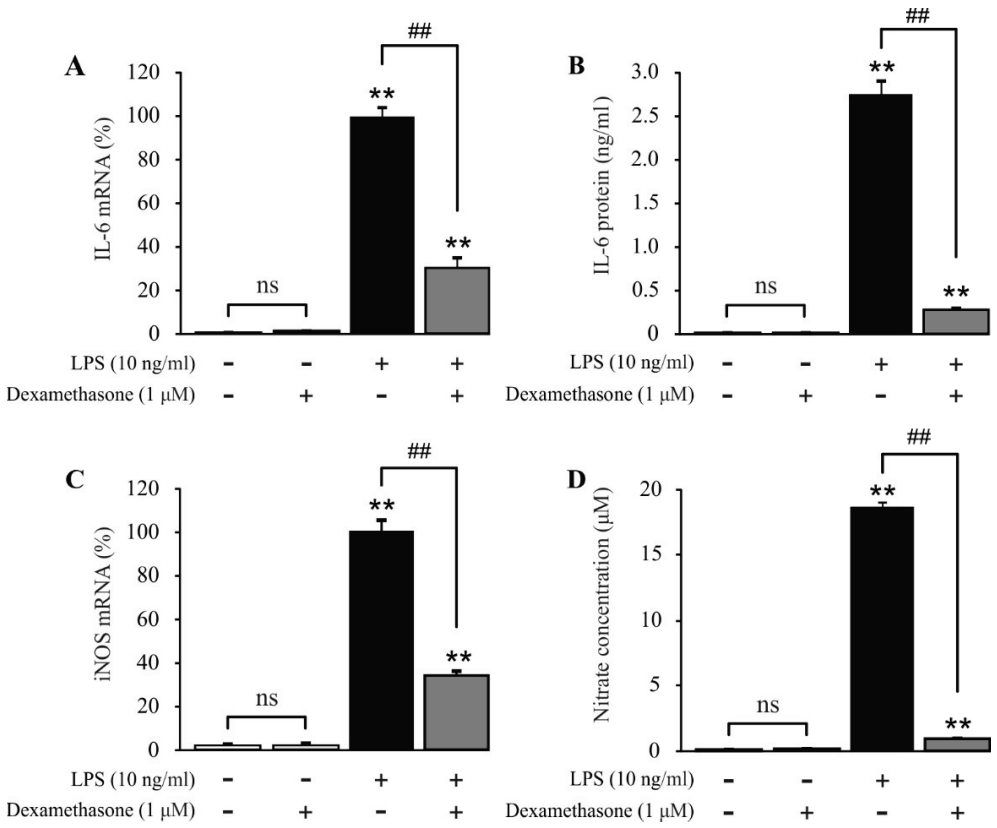

Figure 5-3. Dexamethasone decreased the expression of markers of classical activation in J774 macrophages. J774 cells were cultured with LPS (10 ng/ml) for 4 hours (A and C) or for 24 h (B and D), with or without dexamethasone (1 μM). *Il6* (A) and *iNOS* (C) mRNA levels were measured with RT-qPCR and their expression levels were normalized against *Gapdh*. Expression level in cells treated with LPS alone was set as 100% and the other groups scaled accordingly. IL-6 protein levels were determined with ELISA (B), and NO production was assessed by measuring the concentration of its stable metabolite nitrite by Griess method (D). The results are expressed as mean + SEM, n = 4. **: $p < 0.01$, compared to the untreated control; ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample. Reprinted with permission from Pemmari et al. Basic Clin Pharmacol Toxicol. 2019, 124(4):404-415. Copyright 2018 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

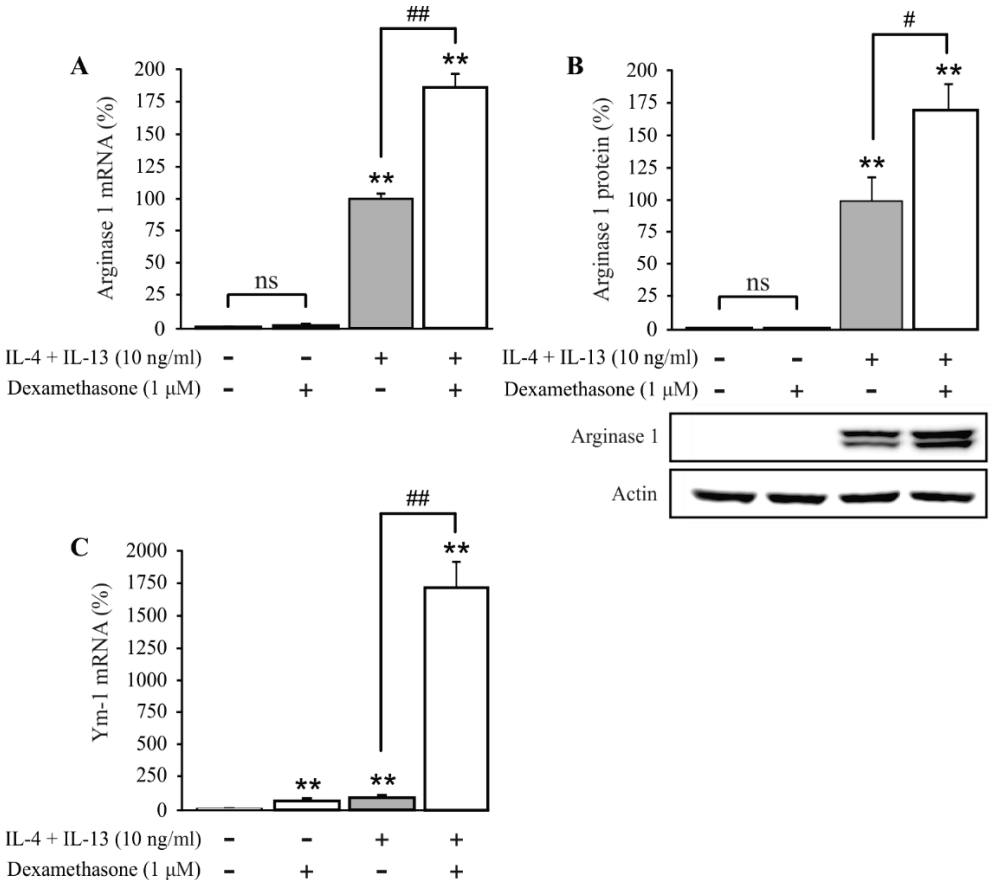

Figure 5-4. Dexamethasone increased the expression of markers of alternative activation in J774 macrophages. J774 macrophages were cultured with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 h, with or without dexamethasone (1 μM). *Argl* (A) and *Ym-1* (C) mRNA levels were measured with RT-qPCR and their expression levels were normalized against *GAPDH*. Arginase 1 protein levels were determined with Western blotting (B). The group treated with IL-4 and IL-13 alone was set as 100% and the other groups scaled accordingly. The results are expressed as mean + SEM, n = 4. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample. In (B), shown is also a representative Western blotting gel of four with similar results. Reprinted with permission from Pemmari et al. Basic Clin Pharmacol Toxicol. 2019, 124(4):404-415. Copyright 2018 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

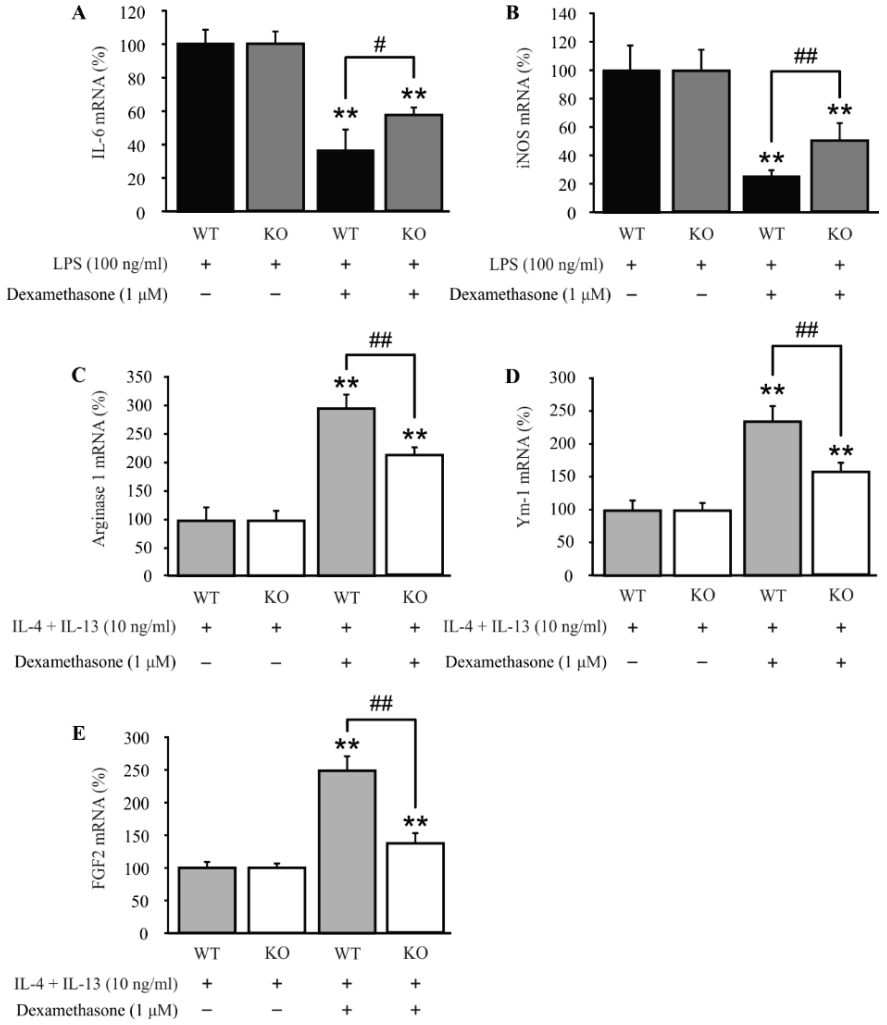

Figure 5-5. Dexamethasone decreased markers of M1 activation and increased those of M2 activation in macrophages from wild-type mice, and both effects were attenuated in macrophages from MKP-1 deficient mice. Peritoneal macrophages from wild-type (WT) and MKP-1 deficient (knock-out, KO) mice were cultured with either LPS (100 ng/ml) for 4 h (A and B) or with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 h (C-E), in the absence or presence of dexamethasone (1 μM). mRNA levels of the M1 activation markers *Il6* (A) and *iNOS* (B) as well as M2 activation markers *Argl* (C), *Ym-1* (D) and *Fgf2* (E) were measured with RT-qPCR and their expression levels were normalized against *Gapdh.* WT and KO values were compared separately to the respective samples without dexamethasone (set as 100%). The results are expressed as mean + SEM, cells from six WT and six KO mice were pooled to give n = 4. **: $p < 0.01$, compared to the control treated with LPS or IL-4 + IL-13 alone; #: $p < 0.05$ and ##: $p < 0.01$, compared to the indicated sample. The experiments were repeated two times with similar results. Reprinted with permission from Pemmari et al. Basic Clin Pharmacol Toxicol. 2019, 124(4):404-415. Copyright 2018 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

To support the role of MKP-1 in the observed effects of glucocorticoids, we studied the effects of dexamethasone on *Mkp-1* expression. Unstimulated peritoneal macrophages from WT mice expressed *Mkp-1* at low levels. When the cells were treated with dexamethasone, *Mkp-1* expression was increased up to five-fold. Dexamethasone also increased *Mkp-1* expression when administered in combination with LPS or IL-4+IL-13. (Figure 5-6, A)

Similar effects were observed in J774 cells (Figures 5-6, B-D). The time-course of *Mkp-1* expression in J774 macrophages (Figure 5-6, B) showed a rapid increase which peaked at 1 h and was followed by another peak at 4 h. This may be postulated to be due to proinflammatory cytokines induced by LPS stimulation or a known feedback loop between MKP-1 and the p38 kinase (Hu et al., 2007; Nimah et al., 2005). Dexamethasone enhanced *Mkp-1* expression both in unstimulated J774 cells as well as in those exposed to LPS or IL-4 + IL-13, and the combination of LPS and dexamethasone was able to induce the highest increase. The effects seen at mRNA level were also translated to protein levels (Figure 5-6, D).

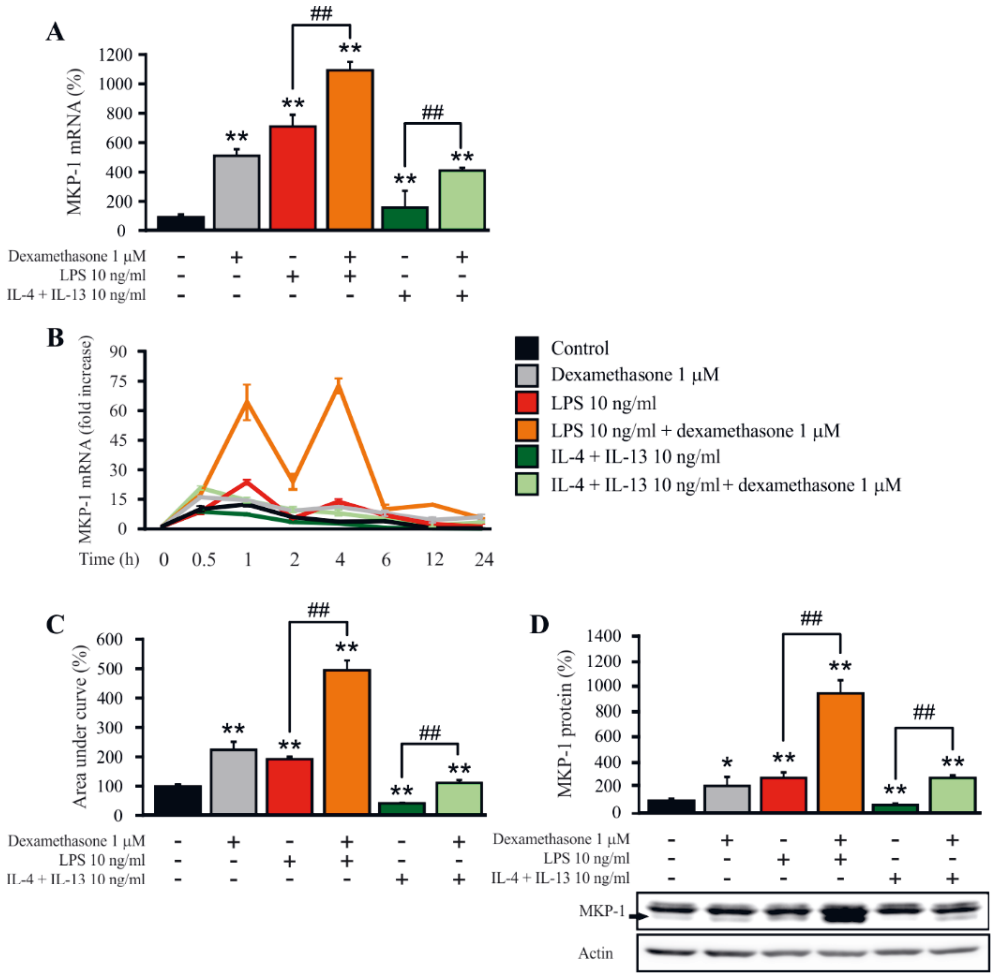

Figure 5-6. MKP-1 expression was increased by dexamethasone in classically activated and alternatively activated macrophages. (A): Peritoneal macrophages from wild-type mice were incubated for 1 h with dexamethasone (1 µM), with LPS (10 ng/ml), with IL-4 and IL-13 (both 10 ng/ml) or with their combinations as indicated, and *Mkp-1* mRNA was detected by RT-qPCR and normalized against *Gapdh*. (B and C): J774 macrophages were incubated for 0.5 – 24 h with dexamethasone (1 uM), with LPS (10 ng/ml), with IL-4 and IL-13 (both 10 ng/ml) or with their combinations as indicated. *Mkp-1* mRNA was detected by RT-qPCR and its expression levels were normalized against *Gapdh*. (B) shows the time-response curves and (C) indicates total area under the curves of (B), estimated by trapezoidal integration. (D): J774 macrophages were incubated for 1 h as described in (A), and MKP-1 protein levels were determined with Western blotting and beta-actin was used as a loading control. The results are expressed as mean + SEM, n = 4. **: $p < 0.01$ and *: $p < 0.05$, compared to the control, ##: $p < 0.01$ for indicated comparisons. In (D), shown is also a representative Western blotting gel of four with similar results. Reprinted with permission from Pemmari et al. Basic Clin Pharmacol Toxicol. 2019, 124(4):404-415. Copyright 2018 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

5.1.3 Nortrachelogenin inhibits alternative macrophage activation and suppresses bleomycin-induced skin fibrosis (II)

Next, we studied the effects of Scots pine (*Pinus sylvestris*) knot extract on alternative macrophage activation. In J774 macrophages, the extract significantly reduced *ArgI* mRNA as compared to the control. Nortrachelogenin, the major lignan constituent of the extract, had a similar effect. This effect was also shared by matairesinol but not by the other major constituents of the extract; on the other hand, pinosylvin significantly increased *ArgI* mRNA (Figure 5-7, A). To investigate whether the effect of nortrachelogenin was shared by the wider lignan family, we investigated the effects of a series of lignans that are previously known as constituents of plant extracts. Again, nortrachelogenin and matairesinol significantly reduced *ArgI* expression in alternatively activated macrophages, but the effect was not shared by hydroxymatairesinol, pinoresinol, secoisolariciresinol or conidendrin (Figure 5-7, B).

The data above suggests that nortrachelogenin significantly contributes to the actions of the pine knot extract in inhibiting M2 activation. Next, we studied the dose-dependence of the effects of nortrachelogenin on *ArgI* expression. As shown in Figure 5-8, both knot extract and nortrachelogenin decreased *ArgI* expression in a dose-dependent manner.

To see whether pine knot extract and nortrachelogenin suppress M2 activation also in human cells, we studied their effects on the expression of *CCL13* and *PDGFB*, which have been recognized as markers of alternative activation in human macrophages. Unstimulated THP-1 macrophages expressed these factors at relatively low levels, and treating the cells with IL-4 and IL-13 for 24 hours significantly increased their expression. In IL-4+IL-13-stimulated THP-1 macrophages, pine knot extract and nortrachelogenin reduced *CCL13* expression in a dose-dependent manner (Figure 5-9). They also inhibited IL-4+IL-13-induced *PDGFB* expression. (Figure 5-10). To study the mechanisms by which pine knot extract and nortrachelogenin affect IL-4+IL-13-induced alternative macrophage activation, we studied their effects on STAT6 phosphorylation and *Pparg* expression. Pine knot extract or nortrachelogenin had no significant effect on STAT6 phosphorylation as measured by Western blotting (Figure 5-11, A). However, both significantly decreased the expression of *Pparg* (Figure 5-11, B) suggesting that the effects of pine knot extract and nortrachelogenin on alternative macrophage activation are, at least partly, mediated through downregulation of PPARγ.

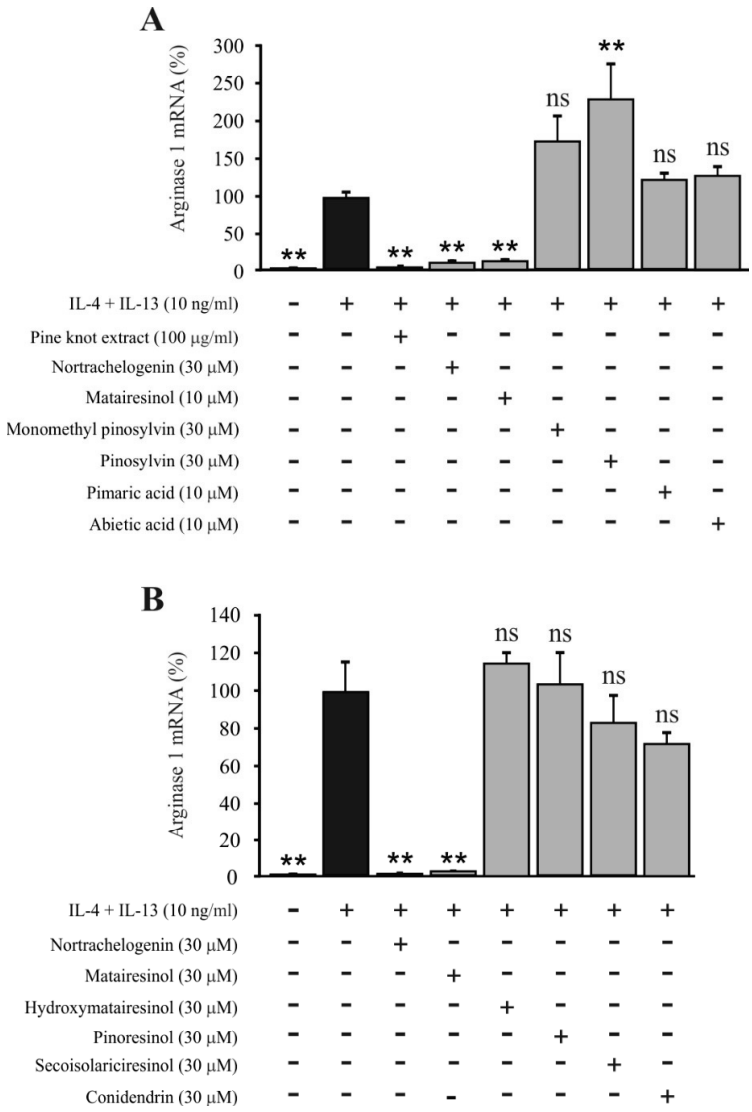

Figure 5-7. Effects of pine knot extract and its major components (A), as well as a series of naturally occurring lignans (B) on ArgI expression in IL-4 and IL-13 -treated alternatively activated macrophages. J774 murine macrophages were cultured for 24 h with IL-4 + IL-13, together with the pine knot extract or its main constituents (used at concentrations close to those present in the extract at 100 µg/ml) (A), or indicated lignans (B). ArgI mRNA levels were determined with RT-qPCR and normalized to Gapdh. Results are shown as mean + SEM, with cells cultured with IL-4 and IL-13 alone set to 100% and the other groups scaled accordingly. n = 3. **p < 0.01, ns = not significant compared with cells cultured with IL-4 and IL-13 alone. Reprinted with permission from Pemmari et al. J Agric Food Chem 2018, 66(51):13405-13413. Copyright 2018 American Chemical Society.

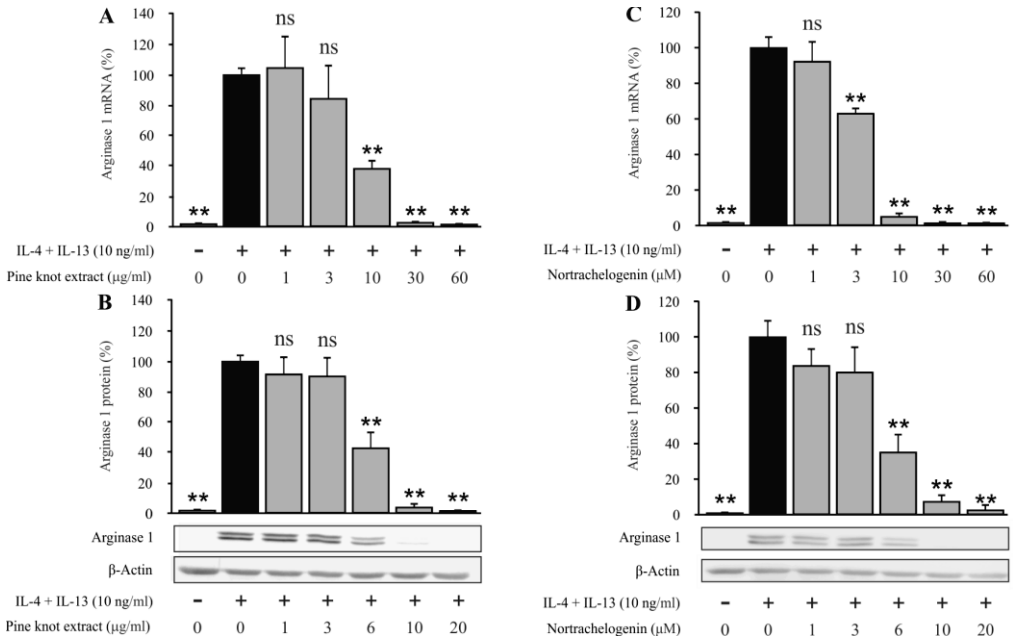

Figure 5-8. Dose-dependent effect of pine knot extract (A-B) and nortrachelogenin (C-D) on *Argl* expression in IL-4 and IL-13 -treated alternatively activated macrophages. Murine J774 macrophages were incubated for 24 h with IL-4 and IL-13, together with increasing concentrations of the pine knot extract (A-B) or nortrachelogenin (C-D). *Argl* mRNA levels were determined with quantitative RT-qPCR and normalized to *Gapdh* (A and C) and protein levels with Western blot, using β-actin as a loading control (B and D). Results are shown as mean + SEM, with cells cultured with IL-4 and IL-13 alone set to 100% and the other groups scaled accordingly. n = 4. **p < 0.01, ns = not significant compared with cells cultured with IL-4 and IL-13 alone. Reprinted with permission from Pemmari et al. J Agric Food Chem 2018, 66(51):13405-13413. Copyright 2018 American Chemical Society.

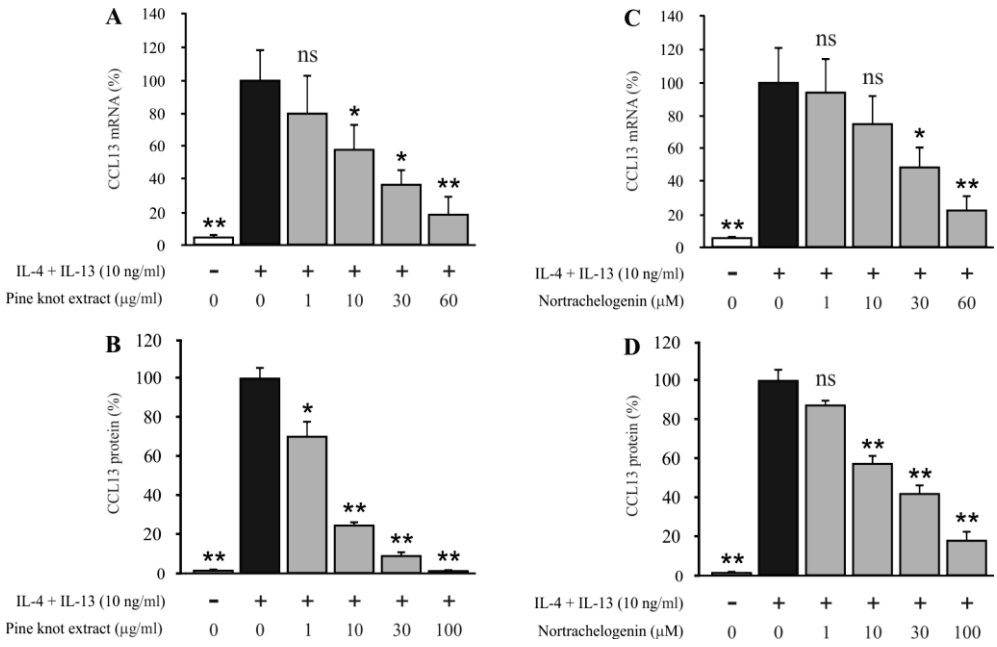

Figure 5-9. Dose-dependent effect of pine knot extract (A-B) and nortrachelogenin (C-D) on *CCL13* expression in IL-4 and IL-13 -treated alternatively activated human macrophages. THP-1 cells were incubated for 24 h with IL-4 and IL-13, together with increasing concentrations of the pine knot extract (A-B) or nortrachelogenin (C-D). *CCL13* mRNA levels, normalized to *GAPDH*, were determined with RT-qPCR (A and C) and protein concentrations in the culture media with ELISA (B and D). Results are shown as mean + SEM, with cells cultured with IL-4 and IL-13 alone set to 100% and the other groups scaled accordingly. n = 4. *p < 0.05, **p < 0.01 and ns = not significant compared with cells cultured with IL-4 and IL-13 alone. Reprinted with permission from Pemmari et al. J Agric Food Chem 2018, 66(51):13405-13413. Copyright 2018 American Chemical Society.

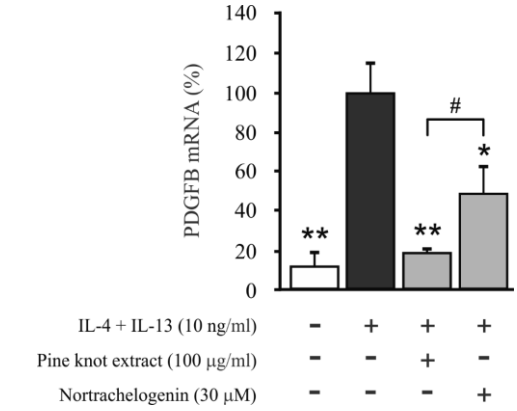

Figure 5-10. Effects of pine knot extract and nortrachelogenin on *PDGFB* expression in IL-4 and IL-13 -treated alternatively activated human macrophages. THP-1 cells were cultured for 24 h in the presence of IL-4 and IL-13, with or without the pine knot extract or nortrachelogenin. *PDGFB* mRNA levels, normalized to *GAPDH*, were determined with RT-qPCR. Results are hown as mean + SEM, with cells cultured with IL-4 and IL-13 alone set to 100% and the other groups scaled accordingly. n = 4. *p < 0.05 and **p < 0.01, compared with cells cultured with IL-4 and IL-13 alone. #p < 0.05 for the indicated comparison. Reprinted with permission from Pemmari et al. J Agric Food Chem 2018, 66(51):13405-13413. Copyright 2018 American Chemical Society.

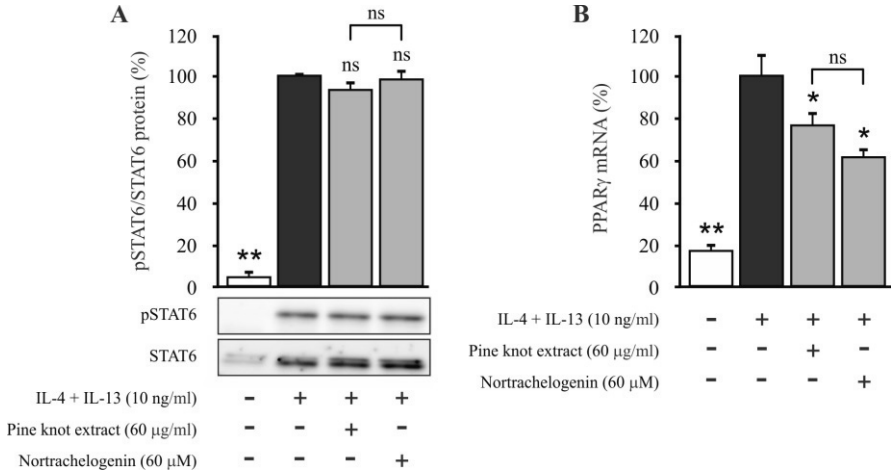

Figure 5-11. Effects of pine knot extract and nortrachelogenin on STAT6 phosphorylation (A) and *Pparg* expression (B) in IL-4 and IL-13 -treated alternatively activated macrophages. Murine J774 macrophages were cultured for 30 min (A) or 2 h (B) in the presence of IL-4 and IL-13, with or without pine knot extract or nortrachelogenin. pSTAT6 protein levels, normalized to total STAT6, were determined with Western blot (A) and *Pparg* mRNA levels, normalized to *Gapdh*, with RT-qPCR (B). Results are shown as mean + SEM, with cells cultured with IL-4 and IL-13 alone set to 100% and the other groups scaled accordingly. n = 4. *p < 0.05 and **p < 0.01, compared with cells cultured IL-4 and IL-13 alone. ns = not significant. Reprinted with permission from Pemmari et al. J Agric Food Chem 2018, 66(51):13405-13413. Copyright 2018 American Chemical Society.

Alternative macrophage activation has been shown to be involved in fibrosing diseases, including a bleomycin-induced murine model of scleroderma. Thus, we decided to study the *in vivo* effects of nortrachelogenin by investigating its effects in this model. Nortrachelogenin was administered subcutaneously to murine skin treated with bleomycin, and skin fibrosis was quantified histologically by determining dermal thickness. Nortrachelogenin partially reversed the increase in skin thickness induced by bleomycin (Figure 5-12, A-B).

Increased collagen production and deposition is a central feature of bleomycin-induced dermal fibrosis. Thus, we evaluated collagen expression in skin after the administration of bleomycin and nortrachelogenin. As illustrated in Figure 5-12, C-E, mRNA levels of collagens *Col1a1*, *Col1a2* and *Col3a1* were enhanced in bleomycin-induced fibrosis, and nortrachelogenin significantly reduced them. Skin expression of *ArgI*, a marker of M2 activation, was also increased by bleomycin. This effect was partially reversed by nortrachelogenin (Figure 5-12, F) suggesting that reduced alternative macrophage activation contributes to the beneficial effect of nortrachelogenin in this model.

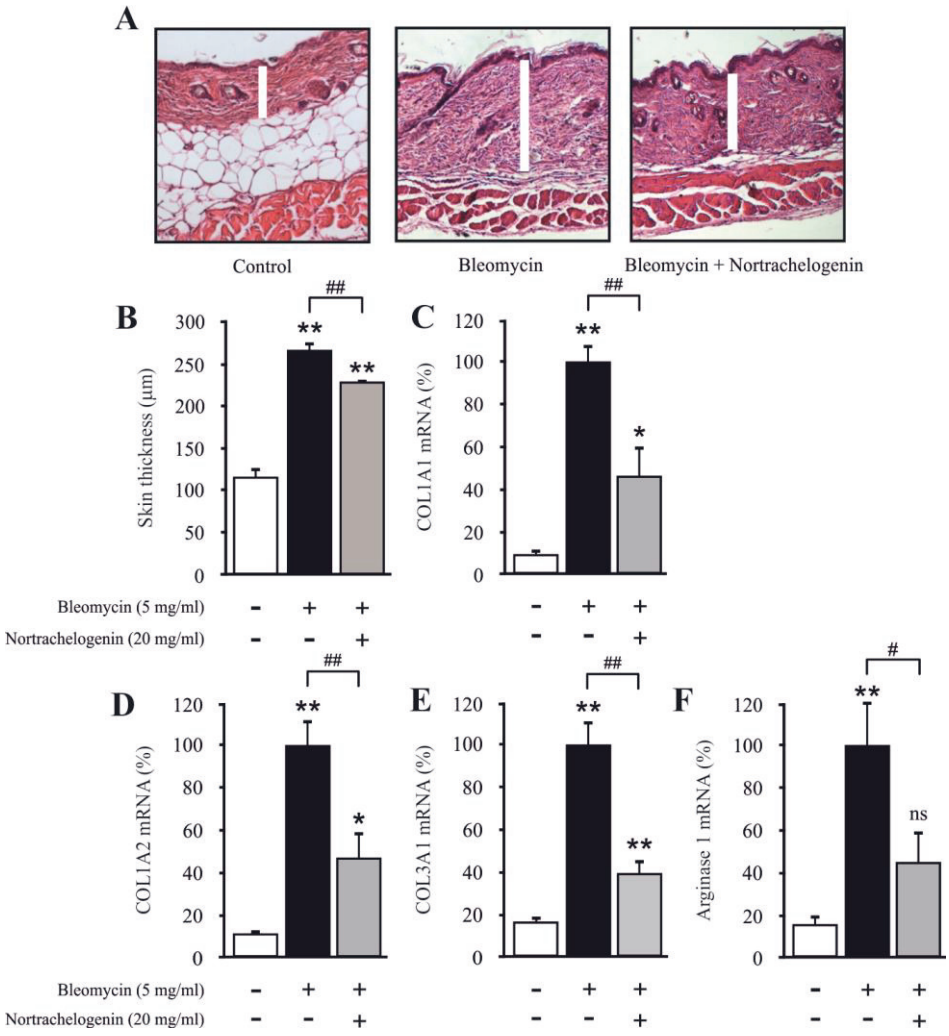

Figure 5-12. Effects of nortrachelogenin on bleomycin-induced dermal fibrosis. A: Representative images of Hematoxylin and Eosin-stained sections of mouse skin. From left to right: control skin, skin treated with bleomycin and skin treated with bleomycin and nortrachelogenin. B: Quantification of dermal thickness. Dermal thickness (μm) was calculated by averaging the thickness measured at 6 different randomly chosen locations in each section using ImageJ program. Bars represent means of the dermal thickness + SEM; n=8. C-F: expression of Col1a1, Col1a2, Col3a1 and Argl (respectively) in control, bleomycin-treated and bleomycin+nortrachelogenin -treated skin. mRNA levels were determined with quantitative RT-qPCR and normalized to Gapdh. Results are shown as mean + SEM, with bleomycin alone set to 100% and the other groups scaled accordingly. n = 7 for bleomycin-treated skin and 8 for others. *p < 0.05, **p < 0.01 and ns = not significant compared with the control. #p < 0.05 and ##p < 0.01 for the indicated comparison. Reprinted with permission from Pemmari et al. J Agric Food Chem 2018, 66(51):13405-13413. Copyright 2018 American Chemical Society.

5.2 Characterization of T_H1 / T_H2 / T_H17 cytokine-induced chondrocyte phenotypes

Next, we sought to test the hypothesis that "polarized" phenotypes analogous to those of T_H cells and/or macrophages can be induced in chondrocytes. Chondrocytes obtained from OA patents were cultured in the presence of the central cytokines associated with different T_H phenotypes (IFNγ, IL-1β, IL-4 and IL-17), total mRNA of the cells was then isolated and sequenced with next generation sequencing (NGS / RNA-Seq).

5.2.1 C(IL-1β) chondrocyte phenotype (III)

IL-1β affected a wide variety of genes in OA chondrocytes. After normalization and correction for multiple testing, a total of 2822 genes were significantly differentially expressed in IL-1β-treated chondrocytes [C(IL-1β) cells] compared with control chondrocytes with a fold change (FC) 2.5 or more in either direction. 1092 of these genes were up- and 1730 downregulated. The 20 most strongly upregulated genes include several proinflammatory cyto- and chemokines, while several genes associated with regulation of gene expression, such as histone proteins, were among the most strongly downregulated ones (Table 5-1).

5.2.2 C(IL-17) chondrocyte phenotype (III)

In chondrocytes treated with the T_H17-associated cytokine IL-17 [C(IL-17) cells], 380 genes were significantly differentially expressed with FC ≥ 2.5 in either direction. 314 of these genes were up- and 66 downregulated. Several genes associated with inflammation and chemotaxis were among the 20 most strongly upregulated genes, while the most strongly downregulated genes included those linked to connective tissue development (Table 5-2).

Table 5-1. C(IL-1β) chondrocyte phenotype: Twenty most strongly up- and downregulated genes in interleukin 1β -treated OA chondrocytes (IL1) relative to control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Gene	Name	Function	Mean (Co)	Mean (IL1)	Fold change	adj. *p*
IL6	Interleukin 6	Inflammation	12.4	18406.9	**3685.72**	< 0.0001
CXCL1	C-X-C motif chemokine ligand 1	Inflammation, chemotaxis	13.8	23793.7	**3457.68**	< 0.0001
IL1B	Interleukin 1 beta	Inflammation	2.8	9575.7	**3332.44**	< 0.0001
CXCL8	C-X-C motif chemokine ligand 8	Inflammation, chemotaxis	329.5	855146.3	**2968.90**	< 0.0001
CXCL6	C-X-C motif chemokine ligand 6	Inflammation, chemotaxis	2.8	4951.8	**2352.02**	< 0.0001
CXCL5	C-X-C motif chemokine ligand 5	Inflammation, chemotaxis	7.4	7352.4	**1239.80**	< 0.0001
CXCL2	C-X-C motif chemokine ligand 2	Inflammation, chemotaxis	3.9	4798.2	**1198.05**	< 0.0001
CXCL3	C-X-C motif chemokine ligand 3	Inflammation, chemotaxis	3.1	3154.6	**1130.76**	< 0.0001
CCL20	C-C motif chemokine ligand 20	Inflammation, chemotaxis	418.0	381100.8	**1128.35**	< 0.0001
IL36RN	Interleukin 36 receptor antagonist	Regulation of inflammation	8.6	5863.8	**914.19**	< 0.0001
ADORA2A	Adenosine A2a receptor	Regulation of inflammation	5.5	1550.7	**641.44**	< 0.0001
IL36G	Interleukin 36 gamma	Inflammation	1.8	1065.5	**562.03**	< 0.0001
EREG	Epiregulin	Regulation of proliferation	31.9	13697.7	**506.87**	< 0.0001
CSF3	Colony stimulating factor 3	Inflammation	0.1	63.9	**300.02**	< 0.0001
VNN1	Vanin 1	T cell migration	9.2	2467.2	**273.35**	< 0.0001
CCL5	C-C motif chemokine ligand 5	Inflammation, chemotaxis	4.1	1134.2	**271.85**	< 0.0001
C15orf48	Chromosome 15 open reading frame 48	Inflammation?	27.2	4669.1	**253.13**	< 0.0001
CCL3	C-C motif chemokine ligand 3	Inflammation, granulocyte activation	0.5	166.3	**242.88**	< 0.0001
FCAMR	Fc fragment of IgA and IgM receptor	Adaptive immunity, leukocyte migration	2.6	492.0	**213.45**	< 0.0001
SERPINB7	Serpin family B member 7	Endoproteinase inhibition	22.1	3747.9	**205.63**	< 0.0001
HRCT1	Histidine rich carboxyl terminus 1	?	105.8	4.1	**-38.85**	< 0.0001
LSP1	Lymphocyte specific protein 1	Regulation of neutrophil mobility	1749.6	58.1	**-31.39**	< 0.0001
HIST1H3G	Histone cluster 1 H3 family member g	Regulation of transcription	183.4	9.6	**-28.26**	< 0.0001
ACTC1	Actin, alpha, cardiac muscle 1	Heart muscle constituent	195.2	10.5	**-24.79**	< 0.0001
NXPH3	Neurexophilin 3	?	39.2	2.4	**-23.89**	< 0.0001
SCN2B	Sodium voltage-gated channel beta subunit 2	Cell adhesion and migration	167.0	8.7	**-22.19**	< 0.0001
HIST1H1A	Histone cluster 1 H1 family member a	Regulation of transcription	908.5	47.2	**-21.20**	< 0.0001
GDF10	Growth differentiation factor 10	Skeletal system development	813.6	45.7	**-20.57**	< 0.0001
LINC02593	Long intergenic non-protein coding RNA 2593	?	68.3	3.4	**-20.53**	< 0.0001
HIST1H3B	Histone cluster 1 H3 family member b	Regulation of transcription	990.6	59.2	**-20.46**	< 0.0001
TMEM26	Transmembrane protein 26	Regulation of proliferation?	403.7	21.4	**-19.30**	< 0.0001
PHYHIPL	Phytanoyl-CoA 2-hydroxylase interacting protein like	Connected to inflammation and ischaemia	22.0	1.6	**-19.19**	< 0.0001
SARDH	Sarcosine dehydrogenase	Mitochondrial metabolism	25.8	2.4	**-19.08**	< 0.0001
HIST1H2BO	Histone cluster 1 H2B family member o	Regulation of transcription?	234.4	12.7	**-18.99**	< 0.0001
ID3	Inhibitor of DNA binding 3, HLH protein	Regulation of transcription	676.5	45.8	**-18.32**	< 0.0001
HIST1H2AJ	Histone cluster 1 H2A family member j	Regulation of transcription?	857.0	47.1	**-18.12**	< 0.0001
HIST1H1B	Histone cluster 1 H1 family member b	Regulation of transcription?	736.0	50.6	**-17.69**	< 0.0001
MFAP2	Microfibril associated protein 2	ECM organization	33.0	3.2	**-17.52**	< 0.0001
TNNT3	Troponin T3, fast skeletal type	Muscle constituent	95.6	6.4	**-17.51**	< 0.0001
HIST1H2AL	Histone cluster 1 H2A family member l	Regulation of transcription?	321.4	21.2	**-17.32**	< 0.0001

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes, blue = downregulated genes.
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Table 5-2. C(IL-17) chondrocyte phenotype: Twenty most strongly up- and downregulated genes in interleukin 17 -treated OA chondrocytes (IL17) relative to control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Gene	Name	Function	Mean (Co)	Mean (IL17)	Fold change	adj. *p*
SAA2	Serum amyloid A2	Chemotaxis	5.5	659.2	**319.99**	< 0.0001
IL6	Interleukin 6	Inflammation	12.2	1431.4	**250.15**	< 0.0001
SAA1	Serum amyloid A1	Inflammation, chemotaxis	63.7	3520.0	**183.26**	< 0.0001
SAA2-4	SAA2-SAA4 readthrough	Chemotaxis?	2.9	216.7	**156.18**	< 0.0001
CXCL6	C-X-C motif chemokine ligand 6	Inflammation, chemotaxis	2.8	276.4	**141.01**	< 0.0001
CXCL1	C-X-C motif chemokine ligand 1	Inflammation, chemotaxis	13.6	1170.5	**136.48**	< 0.0001
VNN1	Vanin 1	T cell migration	9.1	820.5	**84.13**	< 0.0001
CCL20	C-C motif chemokine ligand 20	Chemotaxis	412.8	26508.9	**73.49**	< 0.0001
TNFSF18	TNF superfamily member 18	T cell survival	4.2	470.3	**73.05**	< 0.0001
IL36RN	Interleukin 36 receptor antagonist	Regulation of inflammation	8.5	468.0	**69.09**	< 0.0001
VNN3	Vanin 3	?	1.8	130.3	**66.35**	< 0.0001
ADORA2A	Adenosine A2a receptor	Inflammation, phagocytosis	5.4	105.9	**64.74**	< 0.0001
CXCL2	C-X-C motif chemokine ligand 2	Inflammation, chemotaxis	3.9	220.3	**55.90**	< 0.0001
CXCL8	C-X-C motif chemokine ligand 8	Inflammation, chemotaxis	324.8	14116.5	**48.18**	< 0.0001
C15orf48	Chromosome 15 open reading frame 48	Mitochondrial respiration?	26.9	820.3	**46.34**	< 0.0001
PDZK1IP1	PDZK1 interacting protein 1	Regulation of apoptosis	5.2	206.9	**41.18**	< 0.0001
NOS2	Nitric oxide synthase 2	Inflammation	137.9	3370.2	**40.02**	< 0.0001
ODAPH	Odontogenesis associated phosphoprotein	Enamel production	1.4	41.9	**37.29**	< 0.0001
SLC28A3	Solute carrier family 28 member 3	Nucleoside transport	4.3	150.4	**35.34**	< 0.0001
CXCL5	C-X-C motif chemokine ligand 5	Inflammation, chemotaxis	7.3	207.5	**34.25**	< 0.0001
ACTC1	Actin, alpha, cardiac muscle 1	Cardiac muscle component	191.7	26.7	**-8.14**	< 0.0001
TOX	Thymocyte selection associated high mobility group box	T cell development	14.6	3.9	**-5.66**	0.0010
TMEM26	Transmembrane protein 26	Regulation of proliferation?	396.3	69.8	**-5.47**	< 0.0001
TNNT3	Troponin T3, fast skeletal type	Muscle component	93.9	17.9	**-5.28**	< 0.0001
TENT5B	Terminal nucleotidyltransferase 5B	Regulation of cell proliferation	152.5	39.7	**-4.81**	< 0.0001
TMEM26-AS1	TMEM26 antisense RNA 1	?	32.0	14.4	**-4.77**	0.0004
RCAN2	Regulator of calcineurin 2	Regulation of transcription	326.5	74.6	**-4.74**	< 0.0001
OPRL1	Opioid related nociceptin receptor 1	?	11.8	3.0	**-4.51**	0.0068
CSRNP3	Cysteine and serine rich nuclear protein 3	Regulation of apoptosis	59.7	19.7	**-4.01**	< 0.0001
ASPN	Asporin	Cartilage constituent	2011.2	505.2	**-3.92**	< 0.0001
HRCT1	Histidine rich carboxyl terminus 1	?	104.1	25.8	**-3.85**	< 0.0001
AQP1	Aquaporin 1 (Colton blood group)	Regulation of osmotic pressure, angiogenesis, apoptosis	42.9	13.4	**-3.69**	< 0.0001
YWHAZP5	YWHAZ pseudogene 5	?	10.2	3.2	**-3.68**	0.0133
MRAP2	Melanocortin 2 receptor accessory protein 2	cAMP signaling	1295.9	376.5	**-3.62**	< 0.0001
C1QTNF7	C1q and TNF related 7	?	63.4	20.1	**-3.54**	< 0.0001
MFAP2	Microfibril associated protein 2	Connective tissue organization	32.4	8.7	**-3.47**	< 0.0001
CLEC3A	C-type lectin domain family 3 member A	Skeletal system development	847.3	264.6	**-3.46**	< 0.0001
GREM1	Gremlin 1, DAN family BMP antagonist	Regulation of connective tissue development	5141.6	1566.4	**-3.41**	< 0.0001
CRISPLD1	Cysteine rich secretory protein LCCL domain containing 1	Morphogenesis	946.1	280.2	**-3.39**	< 0.0001
HRASLS5 (=PLAAT5)	HRAS like suppressor family member 5	Glycerophospholipid metabolism	12.8	3.6	**-3.37**	0.0189

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes, blue = downregulated genes.
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5.2.3 C(IFNγ) chondrocyte phenotype (III)

IFNγ significantly affected the expression of a total of 548 genes with FC ≥ 2.5 in either direction. 462 of these were up- and 86 downregulated. In these C(IFNγ) cells, the 20 most strongly upregulated genes included several associated with inflammation and regulation of cell proliferation. Interestingly, several genes involved in antigen processing and presentation were also induced in this phenotype. Genes most strongly downregulated by IFNγ included those involved in cell adhesion, proliferation and migration as well as in Wnt signaling (Table 5-3).

5.2.4 C(IL-4) chondrocyte phenotype (III)

In the C(IL-4) phenotype induced by the T_H2 cytokine IL-4, 26 genes were significantly upregulated with FC > 2.5 compared with control (Table 5-4). There were no genes downregulated by IL-4 to a similar extent, but 10 genes were significantly downregulated with FC < -1.5 (Table 5-5). The upregulated genes included those associated with regulation of inflammation, TGFβ signaling, cell adhesion and metabolism, while several genes linked to cell proliferation were among the downregulated ones.

Table 5-3. C(IFNγ) chondrocyte phenotype: Twenty most strongly up- and downregulated genes in interferon gamma -treated OA chondrocytes (IFNγ) relative to control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Gene	Name	Function	Mean (Co)	Mean (IFNγ)	Fold change	adj. *p*
IDO1	Indoleamine 2,3-dioxygenase 1	Regulation of T cell - mediated immunity	17.5	42320.0	**4643.74**	< 0.0001
LGALS17A	Galectin 14 pseudogene	?	0.4	1065.1	**1750.58**	< 0.0001
GBP1P1	Guanylate binding protein 1 pseudogene 1	Interferon signaling	2.6	2838.8	**1245.34**	< 0.0001
CXCL10	C-X-C motif chemokine ligand 10	Chemotaxis	2.2	2065.2	**1117.91**	< 0.0001
GBP5	Guanylate binding protein 5	Inflammasome activation	1.4	1518.3	**1112.44**	< 0.0001
CXCL9	C-X-C motif chemokine ligand 9	T cell chemotaxis	1.1	1069.9	**1033.80**	< 0.0001
GBP4	Guanylate binding protein 4	Inflammation?	30.9	27565.6	**955.57**	< 0.0001
IFI44L	Interferon induced protein 44 like	Modulation of innate immunity	9.7	6185.8	**694.66**	< 0.0001
GBP1	Guanylate binding protein 1	Negative regulation of inflammation	124.3	54562.1	**454.62**	< 0.0001
HLA-DRA	Major histocompatibility complex, class II, DR alpha	Antigen presentation	5.6	2338.3	**408.93**	< 0.0001
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	Antigen presentation	10.7	2430.7	**383.18**	< 0.0001
CD74	CD74 molecule	Antigen presentation	31.9	11211.5	**353.35**	< 0.0001
RSAD2	Radical S-adenosyl methionine domain containing 2	Antiviral action	44.5	15365.2	**338.82**	< 0.0001
RARRES3	Retinoic acid receptor responder 3	Phospholipid catabolism	33.1	8271.1	**286.40**	< 0.0001
BST2	Bone marrow stromal cell antigen 2	Antiviral action	10.1	2908.5	**285.04**	< 0.0001
GBP6	Guanylate binding protein family member 6	Inflammation	1.0	193.3	**273.26**	< 0.0001
HLA-DRB5	Major histocompatibility complex, class II, DR beta 5	Antigen presentation	4.4	825.4	**253.47**	< 0.0001
HLA-DRB6	Major histocompatibility complex, class II, DR beta 6 (pseudogene)	Antigen presentation?	0.3	125.7	**226.68**	< 0.0001
APOL4	Apolipoprotein L4	Lipid metabolism	2.6	500.8	**225.95**	< 0.0001
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	Regulation of proliferation	96.2	20648.8	**225.79**	< 0.0001
TNFRSF10D	TNF receptor superfamily member 10d	Inhibition of apoptosis	4135.1	501.9	**-7.65**	< 0.0001
ARHGAP9	Rho gtpase activating protein 9	?	10.7	2.4	**-5.27**	0.0028
NANOS1	Nanos C2HC-type zinc finger 1	Regulation of translation and cell migration	83.4	16.9	**-4.94**	< 0.0001
SNORD108	Small nucleolar RNA, C/D box 108	?	66.6	13.8	**-4.81**	< 0.0001
FAM189A2	Family with sequence similarity 189 member A2	?	13.6	4.3	**-4.39**	0.0033
PWAR6	Prader Willi/Angelman region RNA 6	?	34.0	7.9	**-4.32**	< 0.0001
GABRA4	Gamma-aminobutyric acid type A receptor alpha4 subunit	Synaptic transmission	2346.1	549.2	**-4.28**	< 0.0001
CORO2A	Coronin 2A	TLR signaling and Inflammation?	13.5	3.7	**-4.11**	0.0200
WFDC1	WAP four-disulfide core domain 1	Regulation of proliferation	65.1	18.0	**-4.06**	< 0.0001

Table 5-3. (continued)

Gene	Name	Function	Mean (Co)	Mean (IFNγ)	Fold change	adj. *p*
PRSS35	Serine protease 35	?	51.4	13.5	**-4.01**	< 0.0001
SLC16A14	Solute carrier family 16 member 14	Organic acid transport	40.2	13.3	**-3.98**	< 0.0001
PWAR5	Prader Willi/Angelman region RNA 5	?	359.7	91.4	**-3.93**	< 0.0001
MTURN	Maturin, neural progenitor differentiation regulator homolog	?	1857.1	519.7	**-3.63**	< 0.0001
C1QTNF5	C1q and TNF related 5	Cell adhesion	152.4	46.1	**-3.47**	< 0.0001
LONRF2	LON peptidase N-terminal domain and ring finger 2	?	206.8	59.5	**-3.46**	< 0.0001
FGFR4	Fibroblast growth factor receptor 4	Cell proliferation and migration	11.1	5.1	**-3.31**	0.0450
TRABD2B	Trab domain containing 2B	Wnt signaling, proteolysis	14.2	5.5	**-3.29**	0.0014
TNNT3	Troponin T3, fast skeletal type	Muscle contraction	106.0	31.6	**-3.26**	< 0.0001
NCALD	Neurocalcin delta	Endocytosis	17.3	6.6	**-3.24**	0.0290
CDH2	Cadherin 2	Cell adhesion	12.0	4.1	**-3.23**	0.0012

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes, blue = downregulated genes.
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Table 5-4. C(IL-4) chondrocyte phenotype: Twenty most strongly upregulated genes in interleukin 4 -treated OA chondrocytes (IL4) relative to control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Gene	Name	Function	Mean (Co)	Mean (IL4)	Fold change	adj. *p*
KCNK3	Potassium two pore domain channel subfamily K member 3	Potassium ion transport	4.2	41.0	**12.97**	< 0.0001
FGL2	Fibrinogen like 2	Negative regulation of antigen presentation?	26.8	361.2	**10.00**	< 0.0001
SOCS1	Suppressor of cytokine signaling 1	Regulation of inflammation	4.4	37.0	**8.99**	< 0.0001
MAOA	Monoamine oxidase A	Dopamine catabolism	17.8	157.1	**8.56**	< 0.0001
CSN1S1	Casein alpha s1	Steroid signaling?	6.8	20.8	**6.16**	0.0003
LRRC32	Leucine rich repeat containing 32	TGFβ signaling	6.2	36.5	**5.43**	< 0.0001
FAM19A1	Family with sequence similarity 19 member A1, C-C motif chemokine like	Neuroblast differentiation?	4.0	17.5	**5.04**	0.0059
OTOGL	Otogelin like	?	3.8	17.5	**4.86**	0.0003
SUCNR1	Succinate receptor 1	Metabolism and inflammation	3.6	15.1	**4.65**	0.0018
ANPEP	Alanyl aminopeptidase, membrane	Cell adhesion and inflammation	88.3	408.8	**4.41**	< 0.0001
CADPS	Calcium dependent secretion activator	Exocytosis	4.1	11.3	**4.16**	0.0470
SH2D1B	SH2 domain containing 1B	Regulation of inflammation	7.3	27.7	**4.12**	< 0.0001
CISH	Cytokine inducible SH2 containing protein	Regulation of cell growth and inflammation	16.0	64.6	**4.08**	0.0002
SELP	Selectin P	Cell adhesion and inflammation	202.7	849.8	**4.00**	< 0.0001
SAMD3	Sterile alpha motif domain containing 3	Linked to adaptive immunity	4.3	17.3	**3.95**	0.0066
CAPN8	Calpain 8	Linked to rheumatoid arthritis	3.0	10.9	**3.85**	0.0110
LPL	Lipoprotein lipase	Lipid metabolism and inflammation	15.1	44.1	**3.65**	< 0.0001
CYTIP	Cytohesin 1 interacting protein	Cell adhesion	8.9	33.8	**3.61**	< 0.0001
RASL11B	RAS like family 11 member B	TGFβ signaling	4.6	17.9	**3.44**	0.0180
MT-TW	Mitochondrially encoded trna tryptophan	?	24.7	60.6	**3.38**	0.0002

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes.
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Table 5-5. C(IL-4) chondrocyte phenotype: Genes downregulated with FC < -1.5 in interleukin 4 -treated OA chondrocytes (IL4) relative to control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Gene	Name	Function	Mean (Co)	Mean (IL4)	Fold change	adj. *p*
OSR1	Odd-skipped related transciption factor 1	Regulation of cell proliferation	50.2	28.6	**-1.95**	0.0160
ANGPTL7	Angiopoietin like 7	Regulation of angiogenesis	572.7	403.0	**-1.72**	0.0190
SCG5	Secretogranin V	Regulation of protein aggregation	164.7	109.6	**-1.59**	0.0350
OLFML2B	Olfactomedin like 2B	?	84.9	50.7	**-1.56**	0.0250
NEK2	NIMA related kinase 2	Regulation of cell proliferation	420.4	295.1	**-1.55**	0.0002
HIST1H4L	Histone cluster 1 H4 family member l	?	178.3	136.3	**-1.54**	0.0260
NDC80	NDC80, kinetochore complex component	Cell division	821.8	578.4	**-1.53**	< 0.0001
RORC	RAR related orphan receptor C	Regulation of cell differentiation and inflammation?	148.9	105.2	**-1.52**	0.0270
AURKB	Aurora kinase B	Regulation of cell proliferation	164.0	114.5	**-1.52**	0.0044
HMMR-AS1	HMMR antisense RNA 1	Regulation of cell motility?	248.4	173.2	**-1.50**	0.0005

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, blue = downregulated genes.
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5.2.5 Functional description of newly characterized chondrocyte phenotypes and their association with cartilage degradation (III)

Table 5-6 shows the functional gene categories [obtained from the Gene Ontology (GO) database] affected with high significance (p-value < 0.01 when adjusted for multiple comparisons) by at least one of the studied proinflammatory cytokines (IL-1β, IL-17 or IFNγ). The C(IL-1β) phenotype displayed activation of a wide range of inflammatory terms and pathways, along with those related to cell adhesion as well as ECM production and degradation. IL-17 affected a partly overlapping, but smaller, set of inflammatory pathways compared to IL-1β. The C(IFNγ) phenotype again appeared quite distinct from C(IL-1β) and C(IL-17); several terms associated with antigen processing and presentation were affected by IFNγ alone. Nitric oxide synthase biosynthetic process and chemotaxis were among the functions characterizing the C(IL-17) phenotype alone. In addition, many high-level GO terms related to inflammation were affected by all of the three proinflammatory cytokines.

In C(IL-4) cells, no GO terms were significantly enriched among the genes with FC > 2.5 in either direction. When this threshold was lowered to 1.5, several GO terms associated with cell division were among the enriched ones (Table 5-7).

Next, we cross-compared the genes markedly upregulated (FC > 2.5) in C(IL-1β), C(IFNγ) and C(IL-17) chondrocytes to further elucidate the differences and similarities between these phenotypes. Genes markedly upregulated (FC > 2.5) in C(IL-1β) and C(IL-17) chondrocytes had a large amount of overlap; nearly 85% of those upregulated in C(IL-17) cells were also upregulated by IL-1β. However, 45 genes were solely affected by IL-17, and the overlap of C(IFNγ) with the C(IL-1β) and C(IL-17) phenotypes was considerably smaller than that between the two latter phenotypes. Nearly all genes upregulated by both IL-17 and IFNγ were also upregulated by IL-1β (Fig. 5-13A).

When comparing genes markedly downregulated (FC < -2.5) by the three proinflammatory cytokines, the large (>1000 genes) list of genes downregulated in C(IL-1β) chondrocytes again contained a large amount (85%) of those downregulated by IL-17 and a somewhat smaller proportion (48%) of genes similarly affected by IFNγ (Fig. 5-13B).

Table 5-6. GO terms significantly (adj. $p < 0.01$) affected by different proinflammatory cytokines. Genes with FC > 2.5 in either direction were analyzed with DAVID, and the resulting lists were reduced with REVIGO. GO terms significantly affected (with FDR-corrected p-value < 0.05) by a cytokine are marked with an X.

Term	IL1β	IL17	IFNγ	Term	IL1β	IL17	IFNγ
Inflammatory response	X	X	X	Protein heterotetramerization	X		
Immune response	X	X	X	Wound healing	X		
Response to lipopolysaccharide	X	X	X	Regulation of cell proliferation	X		
Chemotaxis	X	X	X	Cell migration	X		
Negative regulation of viral entry into host cell	X	X	X	Regulation of gene silencing	X		
Negative regulation of type I interferon production	X	X	X	Positive regulation of IL-12 production	X		
Response to progesterone	X	X		Odontogenesis	X		
Cell-cell signaling	X	X		Cellular response to mechanical stimulus	X		
Angiogenesis	X	X		Peptidyl-tyrosine phosphorylation	X		
Negative regulation of growth	X	X		Collagen catabolic process	X		
Positive regulation of mitotic nuclear division	X	X		Positive regulation of cell division	X		
Negative regulation of cell proliferation	X	X		Positive chemotaxis		X	
Signal transduction	X		X	Positive regulation of NO synthase biosynthetic process		X	
Response to virus	X		X	Acute-phase response		X	
Positive regulation of IL-6 production	X		X	Positive regulation of cytosolic calcium ion concentration		X	
Response to hydrogen peroxide	X		X	Positive regulation of gtpase activity			X
Positive regulation of IκB/NF-κB signaling	X		X	Response to glucocorticoid			X
Response to drug	X		X	Response to wounding			X
Cellular response to zinc ion		X	X	Positive regulation of NF-κB transcription factor activity			X
Response to toxic substance		X	X	Negative regulation of tumor necrosis factor production			X
Tumor necrosis factor-mediated signaling pathway		X	X	Cellular response to organic cyclic compound			X
Cell division	X			Antigen processing and presentation			X
DNA replication	X			Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II			X
Telomere organization	X			Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent			X
Positive regulation of gene expression	X			Response to IFNα			X
Cell adhesion	X			Response to IFNβ			X
Extracellular matrix organization	X			T cell costimulation			X
Skeletal system development	X			Positive regulation of T cell mediated cytotoxicity			X
Sister chromatid cohesion	X			Defense response			X
DNA replication initiation	X			Protein trimerization			X
Cellular protein metabolic process	X			Proteolysis			X
Cell proliferation	X			Defense response to protozoan			X
Negative regulation of gene expression, epigenetic	X			Positive regulation of peptidyl-tyrosine phosphorylation			X
Nucleosome assembly	X			Protein polyubiquitination			X
Chromosome segregation	X						

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Table 5-7. GO terms enriched among the genes significantly affected by IL-4. The list of genes with FC > 1.5 in either direction and with FDR-corrected *p*-value < 0.05 was analyzed with DAVID, and the resulting list of GO terms was reduced with REVIGO.

Term	**adj. *p***
Cell division	2.45E-11
Chromosome segregation	9.74E-08
Microtubule-based movement	6.36E-06
CENP-A containing nucleosome assembly	0.00035
Protein localization to kinetochore	0.00099
Regulation of gene silencing	0.0014
Metaphase plate congression	0.0018
Protein heterotetramerization	0.0027
G2/M transition of mitotic cell cycle	0.0029
Telomere organization	0.0030
DNA replication-dependent nucleosome assembly	0.0064
Positive regulation of cytokinesis	0.012
Positive regulation of gene expression, epigenetic	0.017
Blood coagulation	0.023
DNA replication	0.026
Anaphase-promoting complex-dependent catabolic process	0.049
Cell proliferation	0.049

adj. *p* = False discovery rate -corrected *p*-value.
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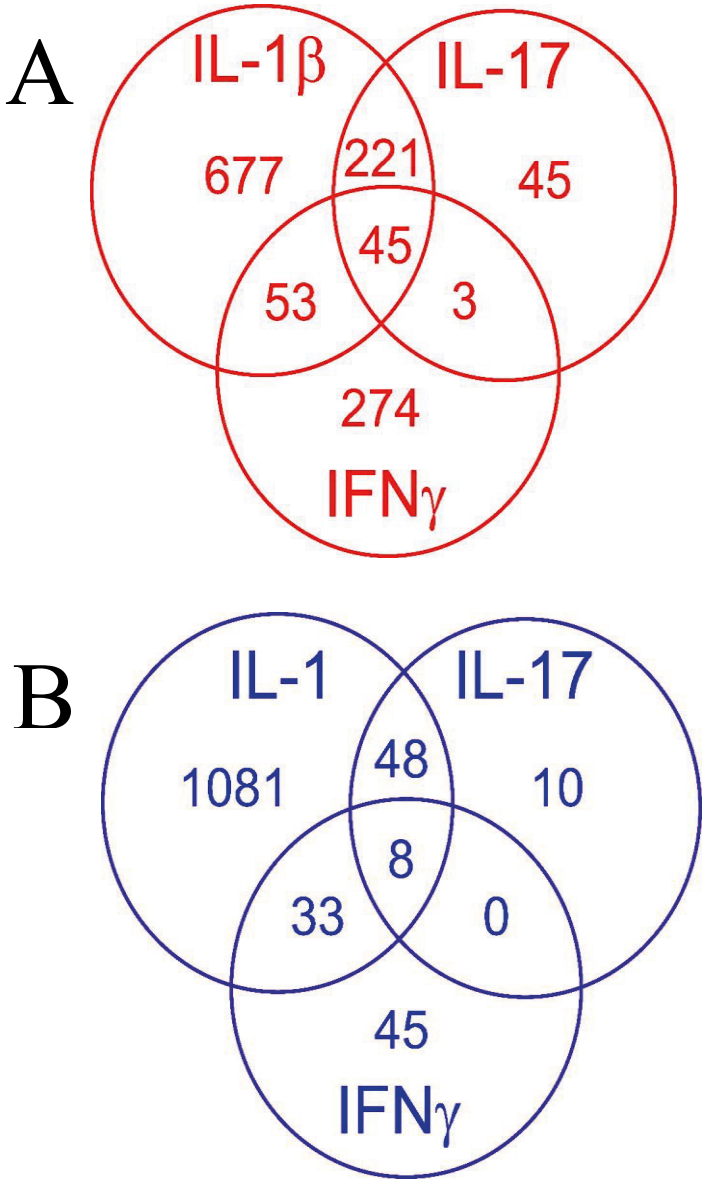

Figure 5-13. Venn diagrams of genes markedly upregulated (FC > 2.5) (A) or markedly downregulated (FC < −2.5) (B) by IL-1β, IL-17 and IFNγ. Reprinted with permission from Pemmari et al. Int J Mol Sci 2021, 22(17):9463.

Some previous genome-wide expression analysis (GWEA) studies have investigated the differences in gene expression between degraded and preserved OA cartilage. Of these, the study by Coutinho de Almeida et al. (2019) is probably the most comprehensive. To see whether IL-1β, IL-17 and IFNγ shift chondrocyte phenotype towards either degraded or preserved cartilage, we compared the genes affected by these cytokines in our data to those identified by Coutinho de Almeida et al. (2019). As a very large number (over 2300) of significantly differentially expressed genes were identified in that study, we first focused on those 84 genes which were most strongly upregulated [FC > 2.5 and false discovery rate (FDR) -corrected p-value < 0.01] in the degraded cartilage. Of those 84 genes, 38 were significantly affected by at least one of the proinflammatory cytokines (IL-1β, IL-17 or IFNγ) in our data. A large majority (30) of these 38 genes were also upregulated by IL-1β, showing that the cytokine shifts chondrocyte phenotype towards the one observed in degraded cartilage. Several mediators of inflammation, such as *LIF*, *CCL20* and *TREM1*, were especially strongly upregulated. Only four of the 84 genes (namely *CLIC3*, *ERFE*, *SLC27A2* and *ANK3*) were downregulated by IL-1β. (Figure 5-14)

In the C(IFNγ) phenotype, 14 of the 84 genes associated with degraded cartilage (including *LIF* and *NGF*) were upregulated compared with control, but nearly as many (nine) were downregulated (including *TREM1*). This shows that the effects of IFNγ on chondrocyte phenotype in relation to the degraded/preserved cartilage are more ambiguous than those of IL-1β. (Figure 5-14)

In C(IL-17) chondrocytes, 25 of the 84 genes associated with degraded cartilage were upregulated compared to naïve chondrocytes (including *CCL20* and *IL11*), and none were significantly downregulated. Nine genes, including *IGFBP1*, *LIF* and *GPR158*, were upregulated in all three inflammatory phenotypes C(IL-1β), C(IFNγ) and C(IL-17) and one (*ANK3*) was downregulated in all of them. (Figure 5-14)

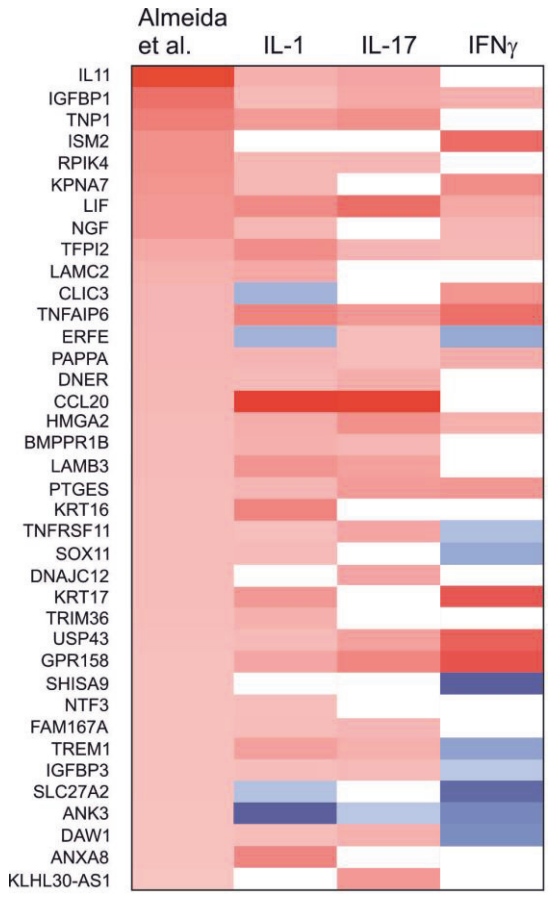

Figure 5-14. Heatmap of genes markedly upregulated (FC > 2.5) in degraded cartilage in the study by Coutinho de Almeida et al. (2019) and significantly affected by at least one studied proinflammatory cytokine. Upregulated genes are marked with red, downregulated with blue, and genes with no significant fold change with white. Reprinted with permission from Pemmari et al. Int J Mol Sci 2021, 22(17):9463.

In Coutinho de Almeida et al. (2019), 52 genes were associated with preserved rather than degraded cartilage (significantly downregulated in degraded cartilage with FC < −2.5). Of these, 19 were significantly affected by at least one of the proinflammatory cyto-kines in our data. In C(IL-1β) cells, 13 of these 19 genes were significantly downregulated with *GDF10* displaying especially strong downregulation. In contrast, five of these genes were upregulated compared to control (including the especially strongly upregulated *C3* and *RSPO3*). This again shows that the net effect of IL-1β is to shift chondrocyte phenotype towards degraded cartilage. IFNγ showed a directionally similar but less pronounced effect: seven of the genes associated with

preserved cartilage were significantly downregulated and three upregulated in the C(IFNγ) phenotype. In C(IL-17) cells, eight genes associated with preserved cartilage were down- and four upregulated; *C3* once again displayed especially strong upregulation. Five genes, including *PTGER3* and *GDF10*, were downregulated in all three chondrocyte phenotypes. On the other hand, *RSPO3* and *PRLR*, both downregulated in degraded compared with preserved cartilage, were upregulated by all of the three cytokines. These data indicate that the C(IL-1β) and C(IL-17) phenotypes at least partly resemble the transcriptomic profile associated with degraded OA cartilage as identified by Coutinho de Almeida et al. (2019). In contrast, IFNγ seems to have a smaller effect on the genes directly linked to cartilage degradation in OA being instead characterized by the upregulation of genes associated with antigen processing and presentation. (Figure 5-15).

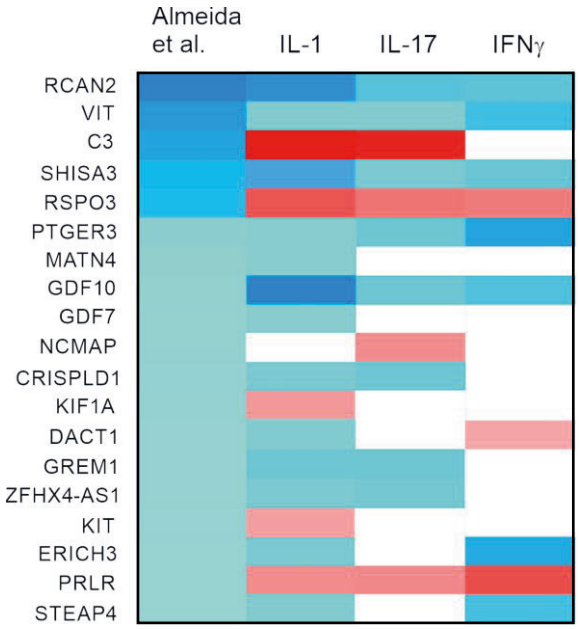

Figure 5-15. Heatmap of genes markedly downregulated (FC < −2.5) in degraded cartilage in the study by Coutinho de Almeida et al. (2019) and significantly affected by at least one studied proinflammatory cytokine. Upregulated genes are marked with red, downregulated with blue, and genes with no significant fold change with white. Reprinted with permission from Pemmari et al. Int J Mol Sci 2021, 22(17):9463.

Relatively few genes were significantly affected by IL-4 in our data, and none of them were markedly (with FC > 2.5) associated with either degraded or preserved cartilage in the data of Coutinho de Almeida et al. (2019). However, looking at genes with a smaller proportional difference between degraded and preserved cartilage (FC > 1.5

in either direction) produced several genes that were significantly affected by IL-4. Ten genes (including *DUSP5*) were upregulated in degraded cartilage and also upregulated in C(IL-4) cells. In contrast, one gene associated with degraded cartilage (*HMMR*) was downregulated by IL-4, and seven genes (including *COL14A1*) associated with preserved cartilage were up-regulated by IL-4. (Table 5-8)

Table 5-8. Genes markedly differentially expressed in degraded and preserved OA cartilage (with FC > 1.5 in either direction) in Coutinho de Almeida et al., 2019 (CdA), and markedly affected (with FC > 1.5 in either direction) by IL-4 in our study.

Gene	Name	FC (CdA)	adj. *p* (CdA)	FC (our study)	adj. *p* (our study)
Genes upregulated in degraded cartilage (CdA et al., 2019) and upregulated by IL-4 (our study)					
PTPRZ1	Protein tyrosine phosphatase, receptor type Z1	**1.94**	0.0190	**2.62**	0.0074
TMEM200A	Transmembrane protein 200A	**1.87**	0.0014	**2.58**	< 0.0001
POSTN	Periostin	**2.06**	0.0320	**2.32**	< 0.0001
TENM3	Teneurin transmembrane protein 3	**1.90**	< 0.0001	**2.08**	< 0.0001
TTC9	Tetratricopeptide repeat domain 9	**2.38**	< 0.0001	**2.07**	< 0.0001
DUSP5	Dual specificity phosphatase 5	**1.51**	0.0310	**1.97**	0.0011
ANGPTL4	Angiopoietin like 4	**1.91**	< 0.0001	**1.67**	< 0.0001
LRRC8C	Leucine rich repeat containing 8 family member C	**2.36**	< 0.0001	**1.65**	0.0003
F5	Coagulation factor V	**1.68**	0.0008	**1.50**	0.0400
COL7A1	Collagen type VII alpha 1 chain	**2.37**	< 0.0001	**1.50**	0.0019
Genes upregulated in degraded cartilage (CdA et al., 2019) and downregulated by IL-4 (our study)					
HMMR	HMMR antisense RNA 1	**1.78**	0.0470	**-1.50**	0.0005
Genes downregulated in degraded cartilage (CdA et al., 2019) and upregulated by IL-4 (our study)					
FGL2	Fibrinogen like 2	**-1.71**	0.0093	**10.00**	< 0.0001
CISH	Cytokine inducible SH2 containing protein	**-2.24**	< 0.0001	**4.08**	0.0002
GPM6A	Glycoprotein M6A	**-1.73**	0.0150	**2.25**	0.0440
COL14A1	Collagen type XIV alpha 1 chain	**-1.75**	0.0330	**2.11**	< 0.0001
CYSLTR1	Cysteinyl leukotriene receptor 1	**-1.68**	0.0009	**1.96**	0.0007
DKK1	Dickkopf WNT signaling pathway inhibitor 1	**-1.66**	0.0180	**1.86**	0.0280
CORO2B	Coronin 2B	**-1.82**	0.0001	**1.60**	0.0140

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes, blue = downregulated genes.
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5.3 Dexamethasone-induced chondrocyte phenotype

In the next part of the thesis, we studied the effects of glucocorticoids on gene expression in OA chondrocytes.

5.3.1 Dexamethasone has a widespread effect on human OA chondrocyte transcriptome (IV)

After normalization and correction for multiple testing, 480 genes were upregulated with a fold change (FC) > 2.0 in dexamethasone-treated cells compared with control, and 755 genes downregulated by the same factor. In total, the expression of 7371 genes was significantly affected by dexamethasone; 3612 of them were up- and 3759 downregulated. Twelve most strongly up- and downregulated genes are listed in Table 5-9. The list of the most strongly upregulated genes includes those associated with e.g. regulation of cell proliferation, cartilage development and regulation of inflammation as well as carbohydrate and lipid metabolism. Markedly downregulated genes include those involved in inflammation, cell differentiation and ECM production. They also include *NGF*, a known central mediator of OA pain (Miller et al., 2017). A list of all genes that were significantly differentially expressed in dexamethasone-treated compared to control chondrocytes can be found in the Supplementary data (Table S2) of Study IV.

Next, we used GO analysis (ranked list enrichment) to study the functional gene categories enriched among the most strongly up- or downregulated genes (Table 5-10). These categories include, among others, those associated with inflammatory response, regulation of cell proliferation and adhesion, ECM organization and collagen synthesis, MAP kinase signaling as well as lipid and carbohydrate metabolism.

Table 5-9. Dexamethasone-induced chondrocyte phenotype: Twelve most strongly up- and downregulated genes in dexamethasone-treated OA chondrocytes (D) relative to control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Gene	Name	Function	Mean (Co)	Mean (D)	Fold change	adj. *p*
GPM6B	Glycoprotein M6B	Regulation of osteoblast function	205.4	8033.4	**37.53**	< 0.0001
SULT1B1	Sulfotransferase family 1B member 1	Sulfation	14.5	702.2	**34.30**	< 0.0001
KLRD1	Killer cell lectin like receptor D1	Regulation of inflammation	15.1	685.0	**29.24**	< 0.0001
FLRT3	Fibronectin leucine rich transmembrane protein 3	Cell adhesion	6.9	247.4	**27.28**	< 0.0001
FAM83A-AS1	FAM83A antisense RNA 1	Regulation of cell proliferation	0.4	43.6	**21.41**	< 0.0001
ZBTB16	Zinc finger and BTB domain containing 16	Cartilage development	113.9	2490.2	**21.11**	< 0.0001
FKBP5	FK506 binding protein 5	Glucocorticoid receptor regulation	702.8	15089.3	**20.11**	< 0.0001
PDK4	Pyruvate dehydrogenase kinase 4	Glucose and lipid metabolism	251.6	5804.9	**19.84**	< 0.0001
PTK2B	Protein tyrosine kinase 2 beta	Regulation of cell proliferation	116.6	2393.8	**19.43**	< 0.0001
ABCD2	ATP binding cassette subfamily D member 2	Lipid metabolism	13.8	340.3	**18.77**	< 0.0001
HAL	Histidine ammonia-lyase	Histidine metabolism	5.5	176.0	**16.34**	< 0.0001
GLUL	Glutamate-ammonia ligase	Cell proliferation	3886.9	64025.9	**15.67**	< 0.0001
PLAU	Plasminogen activator, urokinase	Cell proliferation and migration	169.7	9.5	**-14.03**	< 0.0001
INHBA	Inhibin beta A subunit	Cell proliferation and differentiation	26339.5	2220.8	**-11.71**	< 0.0001
P2RY6	Pyrimidinergic receptor P2Y6	Inflammation	114.3	7.6	**-10.56**	< 0.0001
VCAM1	Vascular cell adhesion molecule 1	Inflammation	24663.5	2854.2	**-8.51**	< 0.0001
GREM1	Gremlin 1, DAN family BMP antagonist	Chondrocyte proliferation and differentiation	6327.8	691.8	**-8.22**	< 0.0001
HAS2	Hyaluronan synthase 2	ECM production	1922.7	234.2	**-8.11**	< 0.0001
SLC26A4	Solute carrier family 26 member 4	Anion transport	1537.7	180.9	**-7.73**	< 0.0001
ARSI	Arylsulfatase family member I	Glycosphingolipid metabolism	102.6	11.7	**-7.46**	< 0.0001
MXRA5	Matrix remodeling associated 5	TGFβ signaling	1100.1	132.2	**-7.31**	< 0.0001
MYBPH	Myosin binding protein H	Cell adhesion	57.6	5.7	**-7.21**	< 0.0001
TSPAN2	Tetraspanin 2	Cell migration	1192.6	154.4	**-7.11**	< 0.0001
NGF	Nerve growth factor	Pain	129.1	16.2	**-6.92**	< 0.0001

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes, blue = downregulated genes.
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Table 5-10. Ranked Gene Ontology (GO) terms significantly affected by dexamethasone

GO term	Description	*p*-value	adj. *p*
GO:0048523	Negative regulation of cellular process	5.50E-12	3.50E-08
GO:0032502	Developmental process	8.97E-12	3.81E-08
GO:0048519	Negative regulation of biological process	1.27E-11	4.05E-08
GO:0030334	Regulation of cell migration	2.03E-11	5.17E-08
GO:0051239	Regulation of multicellular organismal process	9.27E-11	1.69E-07
GO:0032501	Multicellular organismal process	3.43E-10	4.37E-07
GO:0007166	Cell surface receptor signaling pathway	3.29E-10	4.66E-07
GO:0043062	Extracellular structure organization	6.88E-09	6.75E-06
GO:0030198	Extracellular matrix organization	8.64E-09	7.34E-06
GO:0048856	Anatomical structure development	8.63E-09	7.86E-06
GO:0032879	Regulation of localization	9.94E-09	7.91E-06
GO:0009725	Response to hormone	2.06E-08	1.25E-05
GO:0042127	Regulation of cell proliferation	1.99E-08	1.27E-05
GO:0050896	Response to stimulus	6.37E-08	3.12E-05
GO:0040011	Locomotion	4.10E-07	0.000137
GO:0048518	Positive regulation of biological process	6.06E-07	0.000193
GO:0050794	Regulation of cellular process	6.66E-07	0.000197
GO:0040008	Regulation of growth	6.39E-07	0.000199
GO:0048583	Regulation of response to stimulus	1.27E-06	0.000351
GO:0065008	Regulation of biological quality	2.25E-06	0.000584
GO:0043408	Regulation of MAPK cascade	4.7E-06	0.00105
GO:0009719	Response to endogenous stimulus	7.05E-06	0.00152
GO:0006954	Inflammatory response	1.29E-05	0.00235
GO:0006950	Response to stress	1.36E-05	0.00241
GO:0065007	Biological regulation	1.88E-05	0.00281
GO:0051174	Regulation of phosphorus metabolic process	2.16E-05	0.00303
GO:0010273	Detoxification of copper ion	2.21E-05	0.00306
GO:0048878	Chemical homeostasis	2.36E-05	0.0032
GO:0010941	Regulation of cell death	3.27E-05	0.00426
GO:0070482	Response to oxygen levels	4.13E-05	0.00506
GO:0061687	Detoxification of inorganic compound	4.88E-05	0.0057
GO:0006928	Movement of cell or subcellular component	4.99E-05	0.00578
GO:0022610	Biological adhesion	5.45E-05	0.00626
GO:0009611	Response to wounding	5.77E-05	0.0064
GO:0007155	Cell adhesion	6.75E-05	0.00717
GO:0065009	Regulation of molecular function	0.000127	0.0116
GO:0051338	Regulation of transferase activity	0.000334	0.023
GO:0006068	Ethanol catabolic process	0.000349	0.0239
GO:0019216	Regulation of lipid metabolic process	0.000382	0.0255
GO:0009628	Response to abiotic stimulus	0.000579	0.0342
GO:0006109	Regulation of carbohydrate metabolic process	0.000668	0.0375
GO:0051246	Regulation of protein metabolic process	0.000701	0.039
GO:0002682	Regulation of immune system process	0.000804	0.0425
GO:0032964	Collagen biosynthetic process	0.000817	0.0429
GO:0008283	Cell proliferation	0.000895	0.0454

adj. *p* = FDR-corrected *p*-value.

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Chronic low-grade inflammation and oxidative stress as well as changes in ECM production and catabolism are central features in the pathogenesis of OA. Thus, we separately studied genes linked to these processes (Table 5-11). Dexamethasone markedly downregulated several proinflammatory factors such as TNF superfamily member 15 (*TNFSF15*, FC -5.98), *CCL2* (FC -5.43) and *COX-2* (FC -4.29), while the anti-inflammatory MAP kinase phosphatases 1 (*MKP-1*, FC 10.48) and 2 (*MKP-2*, FC 5.50) were upregulated. Genes involved in cellular responses to oxidative stress, such as Krüppel-like factor 9 (*KLF9*, FC 10.85) and forkhead box O3 (*FOXO3*, FC 4.29) were also upregulated. Also, the catabolic matrix metalloproteinases *MMP13* (FC -4.08), *MMP16* (FC -3.12) and *MMP1* (FC -2.85), were downregulated by dexamethasone. Various collagens were downregulated by dexamethasone, including those with the highest expression levels: *COL11A1* (FC -3.12) and *COL2A1* (FC -2.28). However, aggrecan (*ACAN*) was significantly upregulated (FC 2.43). The expression of connective tissue growth factor (*CTGF*, FC 2.25) was enhanced while *FGF1* (FC -2.62), *TGFB2* (FC -2.57) and *VEGFA* (FC -2.36) were downregulated by dexamethasone.

Table 5-11. Selected genes linked to inflammation, oxidative stress, catabolic enzymes, and ECM production as well as growth factors in dexamethasone-treated OA chondrocytes (D) relative to control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Inflammation and oxidative stress					
Gene	**Name**	**Mean (Co)**	**Mean (D)**	**Fold Change**	**adj. *p***
KLF9	Krüppel like factor 9	582.2	6410.9	**10.85**	< 0.0001
MKP-1	MAP kinase phosphatase 1	695.1	7460.0	**10.48**	< 0.0001
MKP-2	MAP kinase phosphatase 2	227.2	1198.1	**5.50**	< 0.0001
TLR2	Toll like receptor 2	120.3	617.0	**4.72**	< 0.0001
FOXO3	Forkhead box O3	1541.3	6657.3	**4.29**	< 0.0001
IRF4	Interferon regulatory factor 4	27.6	128.5	**3.97**	< 0.0001
IL1R1	Interleukin 1 receptor type 1	3690.2	14009.3	**3.81**	< 0.0001
PPARG	Peroxisome proliferator activated receptor gamma	24.5	79.5	**3.12**	< 0.0001
IL16	Interleukin 16	573.4	1724.3	**2.95**	< 0.0001
DUSP5	Dual specificity phosphatase 5	115.6	329.9	**2.79**	< 0.0001
SOD2	Superoxide dismutase 2	40215.6	112308.8	**2.77**	< 0.0001
TNFSF15	TNF superfamily member 15	50.5	6.0	**-5.98**	< 0.0001
CCL2	C-C motif chemokine ligand 2	176.3	29.9	**-5.43**	< 0.0001
FGFR3	Fibroblast growth factor receptor 3	1524.2	338.9	**-4.50**	< 0.0001
COX-2	Cyclooxygenase-2	1232.1	280.9	**-4.29**	< 0.0001
IL6	Interleukin 6	25.8	4.0	**-3.86**	< 0.0001
CX3CL1	C-X3-C motif chemokine ligand 1	30.1	10.6	**-2.58**	< 0.0001
IL11	Interleukin 11	30.4	8.2	**-2.58**	< 0.0001
Catabolic enzymes and their inhibitors					
Gene	**Name**	**Mean (Co)**	**Mean (D)**	**Fold Change**	**adj. *p***
MMP7	Matrix metalloproteinase 7	3.3	29.0	**4.89**	< 0.0001
MMP19	Matrix metalloproteinase 19	9.7	31.3	**2.89**	< 0.0001
MMP2	Matrix metalloproteinase 2	165.6	347.7	**2.06**	< 0.0001
MMP13	Matrix metalloproteinase 13	2452.4	536.6	**-4.08**	0.0003
MMP16	Matrix metalloproteinase 16	1004.9	323.5	**-3.12**	< 0.0001
MMP1	Matrix metalloproteinase 1	26580.9	9513.7	**-2.85**	< 0.0001
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif 9	484.4	1713.8	**3.14**	< 0.0001
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1	4476.2	1022.4	**-4.29**	< 0.0001
ADAMTS3	ADAM metallopeptidase with thrombospondin type 1 motif 3	207.5	70.6	**-2.83**	< 0.0001
TIMP4	TIMP metallopeptidase inhibitor 4	386.8	1717.0	**4.41**	< 0.0001
TIMP3	TIMP metallopeptidase inhibitor 3	14668.7	6429.5	**-2.35**	< 0.0001
Extracellular matrix components					
Gene	**Name**	**Mean (Co)**	**Mean (D)**	**Fold Change**	**adj. *p***
COL13A1	Collagen type XIII alpha 1 chain	51.2	7.2	**-4.99**	< 0.0001
COL27A1	Collagen type XXVII alpha 1 chain	2220.9	468.6	**-4.76**	< 0.0001
COL9A1	Collagen type IX alpha 1 chain	678.5	176.2	**-3.66**	< 0.0001
COL1A1	Collagen type I alpha 1 chain	146.3	46.8	**-3.12**	< 0.0001
COL11A1	Collagen type XI alpha 1 chain	59852.0	19522.6	**-3.12**	< 0.0001
COL5A1	Collagen type V alpha 1 chain	5372.5	1790.5	**-2.95**	< 0.0001

Table 5-11. (continued)

Extracellular matrix components (continued)					
Gene	**Name**	**Mean (Co)**	**Mean (D)**	**Fold Change**	**adj. *p***
COL14A1	Collagen type XIV alpha 1 chain	1474.2	562.4	**-2.69**	< 0.0001
COL16A1	Collagen type XVI alpha 1 chain	991.9	408.0	**-2.39**	< 0.0001
COL15A1	Collagen type XV alpha 1 chain	9376.8	4058.2	**-2.31**	< 0.0001
COL2A1	Collagen type II alpha 1 chain	130240.1	56195.3	**-2.28**	< 0.0001
COL5A2	Collagen type V alpha 2 chain	19215.6	8756.4	**-2.22**	< 0.0001
COL10A1	Collagen type X alpha 1 chain	2211.9	1059.0	**-2.16**	< 0.0001
COL1A2	Collagen type I alpha 2 chain	5477.4	2601.9	**-2.11**	< 0.0001
ACAN	Aggrecan	81094.4	202992.8	**2.43**	< 0.0001
ELN	Elastin	377.4	99.1	**-3.78**	< 0.0001
Growth factors					
Gene	**Name**	**Mean (Co)**	**Mean (D)**	**Fold Change**	**adj. *p***
TDGF1	Teratocarcinoma-derived growth factor 1	4.0	30.4	**5.21**	< 0.0001
CTGF	Connective tissue growth factor	16003.3	36823.5	**2.25**	< 0.0001
PGF	Placental growth factor	73.6	11.4	**-5.13**	< 0.0001
TGFB3	Transforming growth factor beta 3	318.8	63.1	**-4.86**	< 0.0001
NDP	NDP, norrin cystine knot growth factor	259.2	51.5	**-4.72**	< 0.0001
FGF1	Fibroblast growth factor 1	6015.9	2323.5	**-2.62**	< 0.0001
TGFB2	Transforming growth factor beta 2	7649.3	2938.2	**-2.57**	< 0.0001
VEGFA	Vascular endothelial growth factor A	8007.4	3366.9	**-2.36**	< 0.0001
GDF5	Growth differentiation factor 5	474.1	229.3	**-2.11**	< 0.0001

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes, blue = downregulated genes.
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Glucocorticoids are known to regulate carbohydrate and lipid metabolism in many cell types. Also, OA is associated with metabolic alterations in both systemic level (metabolic syndrome) and in the level of chondrocytes. Thus, we set out to separately study genes coding for the proteins participating in the main pathways of carbohydrate and lipid metabolism (glycolysis, oxidative phosphorylation, lipolysis and beta-oxidation). Dexamethasone did not markedly affect (with FC $\geq$ 2.0) the expression of any of these genes except for the upregulation of long-chain acyl-CoA dehydrogenase (*ACADL*) (FC 2.60) (Table S5 in the Supplementary Data of Study IV).

However, dexamethasone markedly affected the expression of several genes regulating carbohydrate and lipid metabolism (those associated with high-level GO terms GO:0006109 Regulation of carbohydrate metabolism and GO:0019216 Regulation of lipid metabolism). For example, pyruvate dehydrogenase kinase 4 (*PDK4*) and glycogen phosphorylase L (*PYGL*), as well as the redox regulator sestrin 3 (*SESN3*), were markedly upregulated by dexamethasone. (Table 5-12 and Figure 5-16)

Table 5-12. Expression of genes regulating carbohydrate (GO:0006109) and lipid (GO:0019216) metabolism in dexamethasone-treated OA chondrocytes (D) compared with control (Co). Mean = average gene expression level in DeSeq2-normalized counts.

Gene	Name	Mean (Co)	Mean (D)	Fold Change	adj. *p*
Regulation of carbohydrate metabolism (GO:0006109)					
PDK4	Pyruvate dehydrogenase kinase 4	251.6	5804.9	**19.84**	< 0.0001
ADRA1B	Adrenoceptor alpha 1B	0.1	23.7	**14.72**	< 0.0001
KCNJ11	Potassium voltage-gated channel subfamily J member 11	0.3	21.6	**12.13**	< 0.0001
CLTCL1	Clathrin heavy chain like 1	104	693.2	**6.45**	< 0.0001
DCXR	Dicarbonyl and L-xylulose reductase	79	196.7	**2.41**	< 0.0001
PYGL	Glycogen phosphorylase L	1785.1	4258.4	**2.38**	< 0.0001
KCNB1	Potassium voltage-gated channel subfamily B member 1	21	54.5	**2.38**	0.0030
IGFBP5	Insulin like growth factor binding protein 5	2688.8	435.7	**-6.07**	< 0.0001
SLC2A12	Solute carrier family 2 member 12	742.4	136.7	**-5.07**	< 0.0001
SOX4	SRY-box 4	1548.9	339.8	**-4.59**	< 0.0001
GALM	Galactose mutarotase	271.7	108.4	**-2.55**	< 0.0001
Regulation of lipid metabolism (GO:0019216)					
ABCD2	ATP binding cassette subfamily D member 2	13.8	340.3	**18.77**	< 0.0001
ABCA6	ATP binding cassette subfamily A member 6	102.4	1070.3	**9.99**	< 0.0001
SORBS1	Sorbin and SH3 domain containing 1	648.8	4163.1	**6.36**	< 0.0001
APOD	Apolipoprotein D	638.3	3819.4	**6.15**	< 0.0001
APOB	Apolipoprotein B	15.7	95.5	**5.16**	< 0.0001
PRKAG2	Protein kinase AMP-activated non-catalytic subunit gamma 2	470.8	1591.1	**3.29**	< 0.0001
PPARG	Peroxisome proliferator activated receptor gamma	24.5	79.5	**3.12**	< 0.0001
SESN3	Sestrin 3	4701.2	14511	**3.1**	< 0.0001
FABP4	Fatty acid binding protein 4	124.8	374.2	**2.93**	< 0.0001
IRS2	Insulin receptor substrate 2	364.5	902.6	**2.39**	< 0.0001
PLIN2	Perilipin 2	875.4	1834.3	**2.07**	< 0.0001
FABP3	Fatty acid binding protein 3	624.2	241.1	**-2.58**	< 0.0001
FABP5	Fatty acid binding protein 5	1357.8	634.5	**-2.13**	< 0.0001

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate -corrected *p*-value, red = upregulated genes, blue = downregulated genes.
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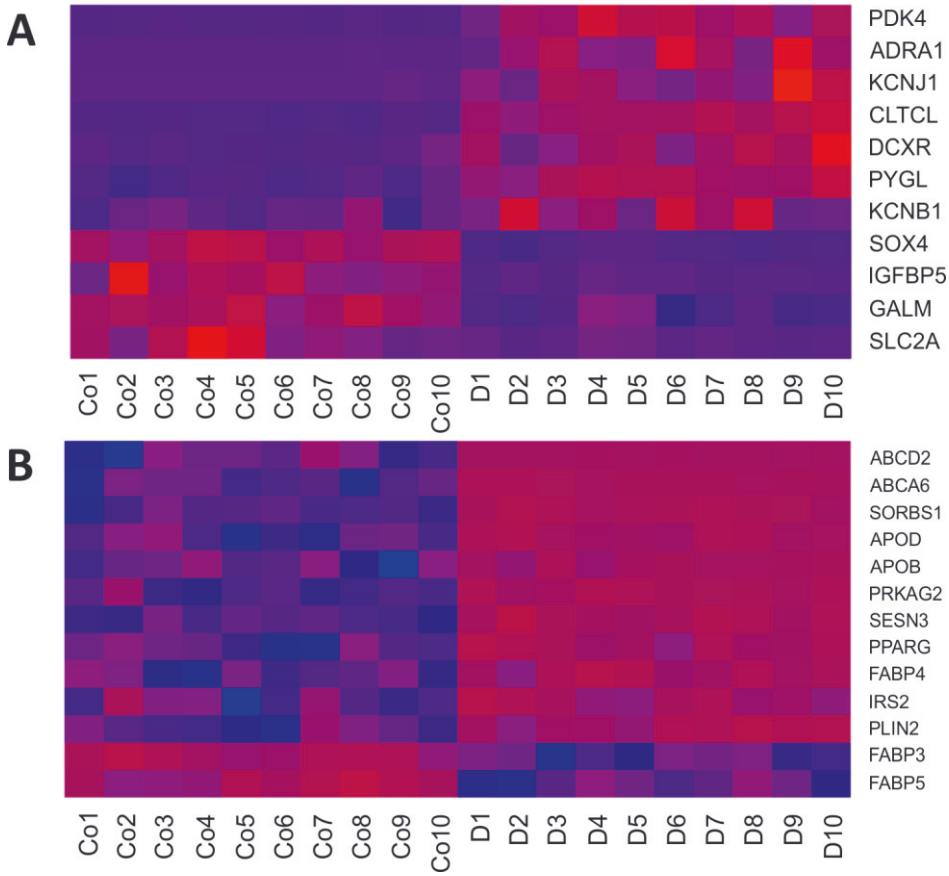

Figure 5-16. Heatmaps showing expression of differentially expressed genes involved in carbohydrate (A) and lipid (B) metabolism, in dexamethasone-treated OA chondrocytes (D) and controls (Co). Expression values are DESeq2-normalized and row scaled, with blue signifying lower and red higher expression. Reprinted with permission from Pemmari et al. Arthritis Res Ther 2020, 22(1):271.

Next, we studied the interactions among the protein products of the genes most strongly affected by dexamethasone (with FC > 5.0 in either direction) using the STRING database. Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3KR1), vascular cell adhesion protein 1 (VCAM1), KIT proto-oncogene receptor tyrosine kinase (KIT), protein tyrosine kinase 2 beta (PTK2B), apolipoprotein B (APOB), FGR proto-oncogene Src family tyrosine kinase (FGR), CCL2 and NGF were found to occupy central positions in the interaction network (Figure 5-17).

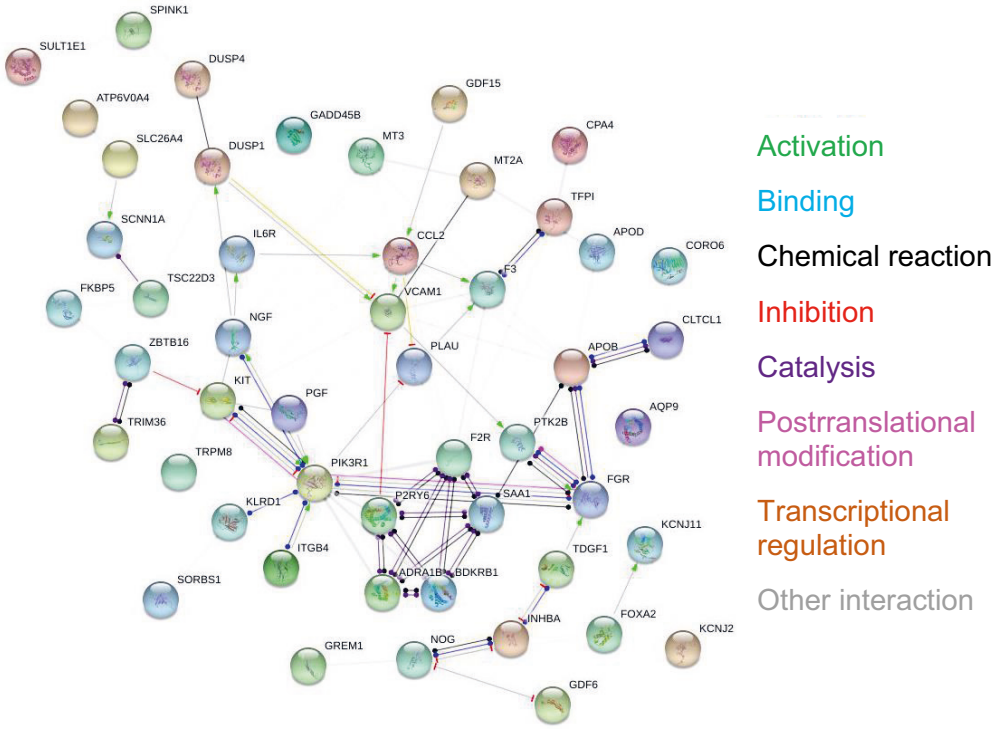

Figure 5-17. Interactions among the protein products of the genes that were up- or downregulated by dexamethasone with an FC 5.0 or greater into either direction, as determined with STRING. Genes with no interactions are excluded from the graph. Colors of the edges: green = activation, blue = binding, black = chemical reaction, red = inhibition, violet = catalysis, pink = posttranslational modification, yellow = transcriptional regulation, grey = other interaction. Reprinted with permission from Pemmari et al. Arthritis Res Ther 2020, 22(1):271.

5.3.2 Dexamethasone partly normalizes OA chondrocyte phenotype (IV)

Genome-wide association (GWAS) studies have identified at least 53 genes associated with OA (Aubourg et al., 2021; Evangelou et al., 2014; Skarp et al., 2018; Wang et al., 2016; Zeggini et al., 2012). When this list of genes was compared with those significantly affected by dexamethasone in our data (with a FC greater than 2.0), 12 overlapping genes were identified (Table S6 of Study IV). Eleven of these genes (including *COL11A1*, *COX-2*, *GDF5*, *IL6*, and *VEGFA*) were downregulated by dexamethasone and only one, *IL16*, upregulated.

Gene expression differences between degraded and preserved OA cartilage have previously been studied using both microarray-based and NGS methods. The microarray-based GWEA study by Ramos et al. (2014) identified 18 genes that were differentially expressed between degraded and preserved OA cartilage, and two of these genes, namely *COL9A1* and *NGF*, were significantly affected by dexamethasone in our data with FC > 2.5 in either direction (Table S7 of Study IV). Of these, the expression of *COL9A1* was previously found to be lower in degraded cartilage compared with preserved, and the gene was also downregulated by dexamethasone. *NGF*, in turn, was expressed at higher levels in degraded cartilage. In our data, it was strongly downregulated by dexamethasone.

Coutinho de Almeida et al. (2019) analyzed gene expression differences between degraded and spared OA cartilage with NGS. In their results, 372 genes were differentially expressed with FC > 2.0 into either direction, and 78 of these genes were markedly affected by dexamethasone (FC > 2.0 into either direction) in the present study. Of these, 25 were upregulated in degraded cartilage compared with spared cartilage and downregulated by dexamethasone in our data, while 19 were upregulated in degraded cartilage and upregulated by dexamethasone. 17 were downregulated in degraded cartilage and upregulated by dexamethasone, while another 17 were downregulated in degraded cartilage and by dexamethasone (Table 5-13). A detailed list of these genes, including their expression levels and FC values, can be found in Study III (Table S8). Interestingly, *NGF* was one of the genes whose expression was increased in degraded OA cartilage and normalized by dexamethasone.

Table 5-13. Comparing the expression fold changes (FC) caused by dexamethasone in our study and Coutinho de Almeida et al. 2019. Genes whose expression is normalized by dexamethasone are **bolded**.

	Downregulated in degraded cartilage	Upregulated in degraded cartilage
Upregulated by dexamethasone	**PDZRN4** **SAA1** **RSPO3** **WISP2** **KIT** **APOD** **METTL7A** **SLC14A2** **CYP4F22** **TRABD2B** **PRLR** **ALDH1L1** **HS6ST3** **AQP7P2** **ACADL** **STC2** **FMO2**	KLRD1 WNT16 CLVS2 DUSP4 TRIM36 SLC7A2 AOC2 DSC3 AFAP1L1 AOC3 DAW1 OR7E14P ZNF474 DSG3 FSTL3 GALNT13 DNER ANK3 CYFIP2
Downregulated by dexamethasone	GREM1 SLC26A4 RFLNA BMPER PIEZO2 COL9A1 SHISA3 SPTSSB LRRTM2 ERICH3 PTGER3 DACT1 ZNF385C CRISPLD1 CNTFR RNF150 ZFHX4-AS1	**INHBA** **TSPAN2** **NGF** **NOG** **MIR31HG** **ATP6V0A4** **IGFBP3** **MYBL2** **BTBD16** **IGFBP1** **LIF** **SHC4** **GRIA2** **SHISA9** **IL11** **C15orf48** **S100A3** **PIMREG** **RIPK4** **UROC1** **SKA1** **TGFBI** **DNAJC22** **BIRC5** **CLIC3**

5.4 Ibuprofen-induced chondrocyte phenotype

5.4.1 Ibuprofen alone has no significant effect on chondrocyte transcriptome (V)

When human OA chondrocytes were cultured with ibuprofen alone (without exogenous cytokines), no significant differences in gene expression were identified between the ibuprofen and control groups after adjusting for multiple comparisons (FDR adjustment).

5.4.2 In inflammatory conditions, ibuprofen affects genes associated with inflammation and cartilage structure (V)

In inflammatory conditions (when OA chondrocytes were cultured with IL-1β), ibuprofen significantly upregulated the expression of 51 genes with FC > 1.5 while 42 were downregulated with FC < -1.5. Fifteen most strongly up- and downregulated genes are shown in Table 5-14. All significantly up- and downregulated genes can be found in Tables S2 and S3, respectively, of the original communication (Study V).

The genes upregulated by ibuprofen included anti-inflammatory factors such as *PPARG* and its coactivator *PPARGC1B* as well as interleukin 10 receptor subunit alpha (*IL10RA*). On the other hand, some genes linked to NO signaling and inflammation, including phosphodiesterase 5A (*PDE5A*), C-X-C motif chemokine receptor 3 (*CXCR3*), selectin E (*SELE*) and granulocyte-macrophage colony stimulating factor (*CSF2*/GM-CSF), were also upregulated (Table 5-14).

Several proinflammatory genes, such as *IL23A* and *IL6*, were downregulated by ibuprofen, as was the procatabolic ADAM metallopeptidase with thrombospondin type 1 motif 6 (*ADAMTS6*). Insulin-like growth factor-binding protein 4 (*IGFBP4*), which regulates chondrocyte proliferation by sequestering IGF (Gruber et al., 2013), was also downregulated. Hyaluronan synthase 1 (*HAS1*) and stanniocalcin 1 (*STC1*), previously shown to be upregulated in inflamed OA synovium (Lambert et al., 2014), were also downregulated by ibuprofen (Table 5-14). *HAS1* produces ECM constituents, but it may also promote inflammation via the production of monocyte-attracting pericellular hyaluronan coats (Jokela et al., 2015; Kultti et al., 2006). *STC1*

may inhibit cartilage development, but it has also been implicated in slowing OA progression (Wu et al., 2006; Wu et al., 2019).

When all genes significantly affected by ibuprofen in the presence of IL-1β were analyzed with Ingenuity Pathway Analysis (IPA), several canonical pathways associated with inflammation and cell adhesion were activated. These include IL-8, integrin and ERK/MAPK signaling pathways as well as cAMP-mediated signaling (Table 5-15). When looking at the individual upregulated genes in these activated pathways, they appear to mostly consist of inflammation-regulating or negative feedback factors (such as *DUSP1*/MKP-1 and *PPARG*/PPARγ) rather than the major proinflammatory mediators/effectors of these pathways (Table S6 of Study V). PTEN signaling was inhibited by ibuprofen. (Table 5-15)

When interactions between the protein products of the most strongly differentially expressed genes (those with FC > 1.5 in either direction) were studied with STRING, *IL6* (downregulated by ibuprofen) was identified as a central node in the interaction network (Figure 5-18). Other genes occupying central places in the network include *PPARG*, *CSF2*, serpin family E member 1 (*SERPINE1*, also known as plasminogen activator inhibitor 1 or *PAI-1*), *IL10RA*, integrin subunit alpha X (*ITGAX*) and *SELE*, all of which were upregulated by ibuprofen.

Table 5-14. Ibuprofen-induced chondrocyte phenotype: Fifteen genes most strongly up- and downregulated by ibuprofen in the presence of IL-1β. Mean = average gene expression level in DeSeq2-normalized counts; Co = control and Ibu = ibuprofen-treated chondrocytes.

Gene	Name	Function	Mean (Co)	Mean (Ibu)	Fold Change	adj. *p*
PPARG	Peroxisome proliferator activated receptor gamma	Carbohydrate and lipid metabolism, inflammation	0.3	0.9	**2.87**	< 0.0001
UMODL1	Uromodulin like 1	Regulation of apoptosis?	0.3	0.7	**2.39**	0.0011
XIRP1	Xin actin binding repeat containing 1	Actin binding	5.7	13.4	**2.38**	< 0.0001
DACT1	Dishevelled binding antagonist of beta catenin 1	Regulation of cell cycle and tissue development	4.0	8.4	**2.10**	< 0.0001
CSF2 / GM-CSF	Colony stimulating factor 2 = Granulocyte-macrophage colony stimulating factor	Leukocyte differentiation, immune response	5.2	11.2	**2.09**	< 0.0001
PPARGC1B	PPARG coactivator 1 beta	Regulation of transcription	0.4	0.8	**2.07**	0.0002
DNM1P35	Dynamin 1 pseudogene 35	?	0.3	0.6	**1.98**	0.0040
LINC00702	Long intergenic non-protein coding RNA 702	Regulation of cell proliferation	3.3	6.3	**1.95**	< 0.0001
FAM186B	Family with sequence similarity 186 member B	?	0.4	0.7	**1.92**	0.0035
SOX17	SRY-box 17	Cell proliferation, tissue development	3.5	6.8	**1.91**	< 0.0001
MTSS1	MTSS1, I-BAR domain containing	Cell adhesion	6.9	12.6	**1.90**	< 0.0001
AKAP6	A-kinase anchoring protein 6	Regulation of cell proliferation, cAMP signaling	2.1	3.9	**1.89**	< 0.0001
PDE5A	Phosphodiesterase 5A	Regulation of NO signaling	1.2	2.3	**1.85**	< 0.0001
RGS2	Regulator of G protein signaling 2	Regulation of G protein signaling	52.1	95.9	**1.85**	< 0.0001
OR7E12P	Olfactory receptor family 7 subfamily E member 12 pseudogene	?	0.5	0.9	**1.81**	0.0110
IL23A	Interleukin 23 subunit alpha	Inflammation	15.2	4.7	**-3.24**	< 0.0001
HAS1	Hyaluronan synthase 1	Extracellular matrix production, inflammation	0.8	0.3	**-2.77**	< 0.0001
IGFBP4	Insulin like growth factor binding protein 4	Cell proliferation and metabolism	213.8	79.7	**-2.73**	< 0.0001
IL6	Interleukin 6	Inflammation	958.4	403.8	**-2.49**	< 0.0001
PDE3A	Phosphodiesterase 3A	Lipid metabolism	0.9	0.3	**-2.48**	0.0001
STAT4	Signal transducer and activator of transcription 4	Inflammation, regulation of cell proliferation	2.5	1.0	**-2.36**	< 0.0001
LINC00968	Long intergenic non-protein coding RNA 968	?	0.7	0.3	**-2.22**	0.0005
PCSK1	Proprotein convertase subtilisin/ kexin type 1	Metabolism	7.2	3.2	**-2.19**	< 0.0001
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif 6	Extracellular matrix catabolism	10.5	4.9	**-2.18**	< 0.0001
HAL	Histidine ammonia-lyase	Histidine catabolism	1.7	0.8	**-2.12**	< 0.0001
DNAH17	Dynein axonemal heavy chain 17	Cytoskeleton component	1.0	0.5	**-2.06**	< 0.0001
CSF3	Colony stimulating factor 3	Inflammation, regulation of cell proliferation	19.8	9.9	**-2.02**	< 0.0001
AREG	Amphiregulin	EGF signaling, regulation of cell proliferation	2.3	1.2	**-2.01**	< 0.0001
CA12	Carbonic anhydrase 12	Acidity regulation, regulation of proliferation	20.9	10.5	**-2.00**	< 0.0001
INSC	Inscuteable homolog (Drosophila)	Cell differentiation	0.6	0.3	**-1.98**	0.0011

Gene functions are obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
adj. *p* = False discovery rate-adjusted *p*-value, red = upregulated genes, blue = downregulated genes.
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Table 5-15. Canonical IPA pathways significantly up- or downregulated (z-score ≥ 2.5 or ≤ -2.5) by ibuprofen in the presence of IL-1β. All genes significantly affected (adj. $p \leq 0.05$) by ibuprofen were included in the analysis.

Canonical pathway	adj. *p*	z-score
Integrin signaling	< 0.0001	**4.95**
Actin cytoskeleton signaling	0.0022	**4.24**
PI3K signaling in B lymphocytes	0.0003	**3.44**
Agrin interactions at neuromuscular junction	0.0037	**3.32**
IL-8 signaling	< 0.0001	**3.29**
ERK5 signaling	0.0083	**3.16**
Glioblastoma multiforme signaling	< 0.0001	**3.14**
Paxillin signaling	< 0.0001	**3.05**
ErbB2-ErbB3 signaling	0.0290	**3.00**
FcγRIIB signaling in B lymphocytes	0.0250	**3.00**
Renal cell carcinoma signaling	0.0016	**3.00**
Bladder cancer signaling	< 0.0001	**3.00**
14-3-3-mediated signaling	0.0058	**2.89**
PKCθ signaling in T lymphocytes	0.0300	**2.84**
Calcium signaling	0.0083	**2.84**
Thrombin signaling	0.0025	**2.83**
CREB signaling in neurons	0.0019	**2.83**
HGF signaling	< 0.0001	**2.83**
Non-small cell lung cancer signaling	0.0029	**2.83**
α-adrenergic signaling	< 0.0001	**2.71**
Endothelin-1 signaling	0.0098	**2.68**
Mouse embryonic stem cell pluripotency	0.0052	**2.67**
NF-κB activation by viruses	0.0009	**2.67**
Macropinocytosis signaling	< 0.0001	**2.67**
CXCR4 signaling	0.0048	**2.67**
p70S6K signaling	0.0026	**2.67**
cAMP-mediated signaling	0.0034	**2.56**
ErbB4 signaling	0.0140	**2.53**
Chemokine signaling	0.0130	**2.53**
Actin nucleation by ARP-WASP complex	0.0008	**2.53**
Regulation of cellular mechanics by calpain protease	< 0.0001	**2.53**
Synaptic long term potentiation	0.0001	**2.52**
Cardiac hypertrophy signaling	0.0002	**2.50**
ERK/MAPK signaling	< 0.0001	**2.50**
fMLP signaling in neutrophils	0.0012	**2.50**
PAK signaling	0.0001	**2.50**
Rac signaling	0.0260	**2.50**
IL-3 signaling	0.0018	**2.50**
Acute myeloid leukemia signaling	0.0017	**2.50**
Telomerase signaling	0.0011	**2.50**
Wnt/Ca+ pathway	< 0.0001	**2.50**
PTEN signaling	0.0009	**-2.67**

adj. p = False discovery rate -adjusted p-value.
z-score = IPA z-score, red = upregulated genes, blue = downregulated genes.
Table reprinted with permission from Pemmari et al. RMD Open 2021, 7(3):e001657.

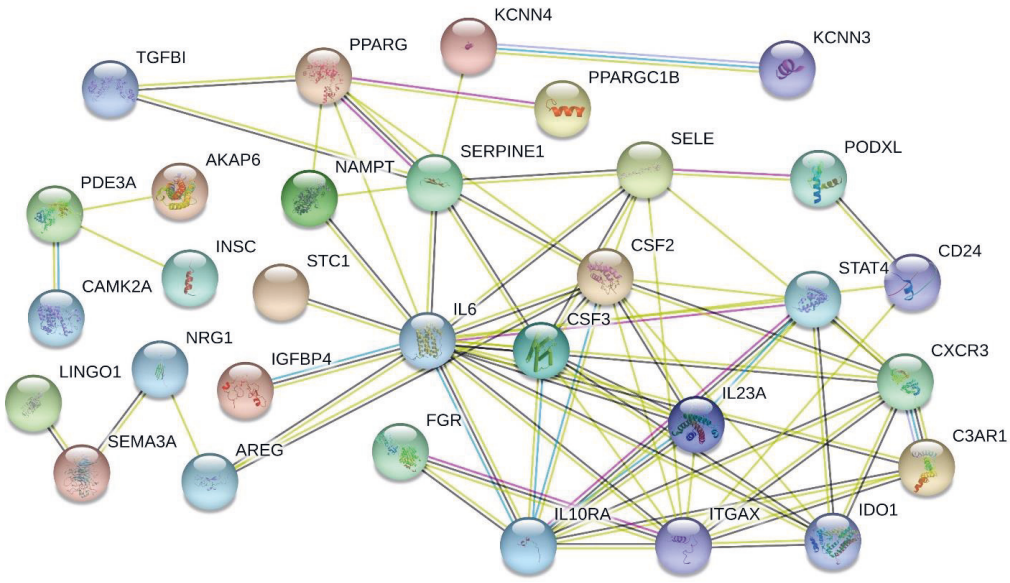

Figure 5-18.Interactions among the genes that were up- or downregulated by ibuprofen with an FC 1.5 or greater in IL-1β -treated cells. Genes with no identified interactions are excluded from the graph. Colors of the edges: green = activation, blue = binding, black = chemical reaction, red = inhibition, violet = catalysis, pink = posttranslational modification, yellow = transcriptional regulation, grey = other interaction. Reprinted with permission from Pemmari et al. RMD Open 2021, 7(3):e001657.

6 DISCUSSION

This thesis established that distinct proinflammatory classical ("M1") and healing-promoting alternative ("M2") phenotypes can be induced in the murine J774 and human THP-1 cell lines as well as in primary murine macrophages. Furthermore, it was shown that certain plant polyphenols inhibit the alternative macrophage phenotype and that the phosphatase MKP-1 can "shift" macrophage phenotype from the classical towards the alternative.

Moving to another cell type playing an important role in inflammatory arthritis, we generated a hypothesis that the central $T_H1/T_H2/T_H17$ cytokines can induce distinct chondrocyte phenotypes resembling the "polarized" phenotypes of T_H cells and macrophages. The resulting phenotypes were characterized by RNA-Seq. The C(IL-1β) phenotype was marked by a widespread induction of proinflammatory and catabolic factors, while a smaller subset of inflammatory genes was upregulated in C(IL-17) cells. C(IFNγ) was identified as a distinct, somewhat proinflammatory phenotype characterized by upregulation of antigen-presenting factors. The effects of IL-4 were much more modest, with some anti-inflammatory factors being upregulated in C(IL-4) cells.

In the next parts of the study, we investigated the effects of two widely used drugs, glucocorticoids (GCs) and nonsteroidal anti-inflammatory drugs (NSAIDs), on the chondrocyte phenotype. GCs are used intra-articularly in the treatment of OA exacerbations, but their long-term effects are controversial. The GC dexamethasone had wide-ranging effects on chondrocyte phenotype in cells isolated from OA patients; it reduced the expression of several inflammatory effectors while upregulating anti-inflammatory factors. Dexamethasone also affected a wide variety of cartilage extracellular matrix constituents, as well as genes involved in glucose and lipid metabolism. NSAIDs are used perorally for arthritis pain, but, as with GCs, there have been some concerns about their long-term effects on cartilage. We found that when given alone, ibuprofen had no significant effect on chondrocyte gene expression. In inflammatory conditions (in cells treated with the proinflammatory cytokine IL-1β), however, ibuprofen upregulated anti-inflammatory factors and

genes associated with cell adhesion while downregulating several mediators of inflammation.

6.1 Methodology

Most of the methods used in this thesis are commonly used and standardized laboratory procedures in biomedical and pharmacological research. The bioinformatic methods used in Studies III–V are, so far, less commonly used in pharmacology. However, relatively well standardized workflows for RNA-Seq studies have also been established (Chatterjee et al., 2018; Ji et al., 2018). This simplifies the interpretation of the results and enables comparing them to the previously published results.

6.1.1 J774 and THP-1 cell lines

In Studies I and II, two immortalized macrophage cell lines were used: murine J774 and human THP-1 cells (Ralph et al., 1975; Tsuchiya et al., 1980). Immortalized cell lines have a relatively stable gene expression over time (Carter & Shieh 2015), increasing the reproducibility of experimental results. However, there is the question of how well these cell lines cultured *in vitro* resemble analogous cell types in a living body. To increase confidence in the current results, primary murine macrophages were also used in Study I.

6.1.2 Primary human OA chondrocytes

Studies III-V used primary OA chondrocytes isolated from cartilage samples received from patient donors undergoing joint replacement surgery. This has several advantages compared with chondrocyte cell lines: the cells can be expected to better resemble *in vivo* chondrocytes in gene expression, lacking the changes brought on by the immortalization process used in creating the cell line. Also, chondrocytes from OA patients have been in contact with synovial fluid containing inflammatory mediators and may thus display gene expression changes caused by the disease, something that can't be fully replicated *in vitro* with line cells. Issues with primary human chondrocytes include relatively rapid dedifferentiation which causes cultured chondrocytes to gradually lose their typical gene expression profiles; relatively short

culture and incubation times (24 hours each) were used in the current study to counteract this. Another potential issue is that chondrocytes are naturally embedded in cartilage extracellular matrix, and interactions with the ECM are an important determinant of chondrocyte wellbeing and function. Culture systems with matrix-mimicking biomaterials have been devised, but these are more complex and introduce their own problems (Campos et al. 2019, Demoor et al., 2012). Yet another issue is genetic and transcriptomic heterogeneity between cartilage donors. In our study, this was accommodated by using paired data analysis (comparing each treated sample to the control sample from the same donor).

6.1.3 Laboratory methods and reagents

This thesis used well-established methods in cell culture as well as protein and RNA isolation and analysis. These methods are widely used and standardized, and the needed reagents are commercially available. Western blot (WB) is a method for separating cellular proteins according to their size. After the separation, labeling the proteins with an antibody followed by appropriate detection methods allows their amount to be determined. WB is relatively inexpensive and rather specific. However, the proteins must be present at sufficient amounts for the method to be able to detect them, and the method is only semiquantitative (allowing the comparison of relative protein abundances between two samples rather than the determination of absolute abundance). Also, issues with antibody specificity can present a problem to WB. ELISA uses antibodies to capture and accurately quantify proteins present in a solution (such as a cell culture medium). When using ELISA on culture medium, the proteins must be excreted from the cells into the media for this method to work, and nonspecific antibodies can once again present a problem. Quantitative reverse transcription polymerase chain reaction is used to quantify the amount of mRNA coding a certain protein in a cell. Using a (presumably) stably expressed "housekeeping" gene as a reference allows for comparing the expression levels of a certain gene between samples. An issue with RT-qPCR is that it only gives information on the amount of mRNA rather than that of the corresponding protein product.

6.1.4 Animal models

MKP-1 KO mice, as well as corresponding WT mice, were used as a source of primary macrophages in Study I. For ethical reasons, the number of mice used was kept in a minimum deemed to be enough for obtaining statistically reliable results.

In Study II, a model of bleomycin-induced skin fibrosis in a mouse was used to study the effects of nortrachelogenin on a model associated with alternative macrophage activation; this was done to investigate if the previously observed *in vitro* results were also translated to an *in vivo* situation. The model involves injecting bleomycin into the mouse skin, causing a fibrotic reaction mimicking conditions such as scleroderma. The bleomycin model has been shown to replicate many of the hallmarks of fibrotic skin diseases, such as increased collagen deposition and alternative macrophage activation (Joshi et al., 2017). However, some of the underlying molecular mechanisms of bleomycin-induced injury, such as the role of oxidative stress, are somewhat controversial (Williamson et al., 2015). In the light of this, replicating the results in another fibrosis model, such as fibrillin 1 (*FBLN1*) tandem duplication (Siracusa et al., 1996), would further elucidate the effects of nortrachelogenin on skin fibrosis.

6.1.5 Next generation sequencing and bioinformatics

The NGS/RNA-Seq workflow used in Studies III-V is fairly well standardized and it was used in cooperation with, and according to the instructions of, a central bioinformatics core facility in Finland [Institute for Molecular Medicine Finland (FIMM), Helsinki]. RNA-Seq facilitates the simultaneous quantification of essentially all mRNA present in a sample. Operating as a massively parallel mRNA multiplication and sequencing process, it shares many advantages and disadvantages with RT-qPCR. However, in addition to its relatively high cost, RNA-Seq also presents unique problems with data analysis. First, reconstructing the transcriptome from sequenced RNA fragments (reads) obtained from the method is not straightforward, and various potential biases and composition effects must be considered. Second, a very large number (usually tens of thousands) of transcripts analyzed simultaneously necessitates adjustments for multiple testing. Third, the sheer volume of data generated from RNA-Seq complicates the interpretation of the results, as biologically relevant and meaningful gene expression changes must often be picked from among a large number of differentially expressed genes. (Finotello

& Di Camillo, 2015) Databases containing the functions of, and interactions between, genes have been developed to facilitate functional interpretation of RNA-Seq results. Gene Ontology (GO), a well-known and well-established database for functional genome annotation was used in our study to identify functional gene categories enriched among the differentially expressed genes. The STRING tool was employed for analyzing the interactions between the protein products of differentially expressed genes, and Ingenuity Pathway Analysis (IPA), a comprehensive proprietary analysis tool, was used to identify biochemical pathways that were activated or inhibited by the studied medications and cytokines (Szklarczyk et al., 2015; The Gene Ontology Consortium, 2017; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). Also, confirming the expression changes of selected differentially expressed genes identified with RNA-Seq with RT-qPCR serves as an important technical "sanity check" for the more complicated sequencing method. Different groups of patients were used for PCR validations to simultaneously confirm the results "out of the sample".

6.2 Macrophage phenotypes in inflammation

6.2.1 The M1/M2 model of macrophage polarization

The classical/alternative (M1/M2) paradigm of macrophage activation was first introduced in the literature over 20 years ago (Goerdt et al., 1999). The initial model was relatively simple, analogous to the proinflammatory T_H1 and anti-inflammatory T_H2 cells. However, more recent studies have questioned this simplified scheme. It is increasingly being recognized that macrophage phenotypes exist in a "spectrum" of different phenotypic markers in different combinations, affected by environmental signals (Mosser & Edwards, 2008). The role of factors such as cellular metabolism (controlled by e.g. mTor signaling) behind the phenotypic changes is also being increasingly appreciated (Kang & Kumanogoh, 2020). However, the somewhat more specific phenotypes of LPS-induced proinflammatory classical activation and IL-4+IL-13-induced alternative activation, which can be thought as the "ends" of the phenotypic spectrum, can be delineated with modern sequencing technologies and thus can be considered (patho)physiologically important (Orecchioni et al., 2019). These phenotypes were also used in this thesis. The M17 phenotype induced by IL-17 is much less well-established compared to the M1/M2

scheme. However, these cells have been reported to have proinflammatory effects in the initial stages of inflammation and later participate in the clearance of apoptotic cells and the resolution of inflammation (Raucci et al., 2022; Zizzo & Cohen, 2013).

A factor influencing the clinical implications of the present results in the setting of arthritis is the degree of similarity between peritoneal and synovial macrophages. To our knowledge, there is no comprehensive data comparing, for example, the gene expression profiles of these two cell populations. However, the resident macrophage populations in synovium and peritoneum are assumed to share a similar origin, primarily deriving from early embryonic haematopoiesis (Cassado et al, 2015; Culemann et al., 2019). This suggests that the phenotypes of these two macrophage populations may be at least somewhat similar.

6.2.2 Effects of MKP-1 and glucocorticoids on macrophage phenotype

MKP-1 is known to act as an important endogenous regulator of inflammation, and this has previously been shown to be mediated at least partly via its effects on inflammatory gene expression in macrophages. Increased production of inflammatory mediators in MKP-1-deficient macrophages was confirmed in this thesis (Study I). Previously, MKP-1-induced inhibition of inflammatory responses has also been confirmed *in vivo*: animals deficient in this gene are more prone to hyperinflammatory septic disease and mortality when challenged with pathogens (Korhonen & Moilanen, 2014; Rodriguez et al., 2010). We extended these previous findings by showing that *MKP-1* deficiency also has a significant effect on the anti-inflammatory alternative macrophage phenotype: classical activation was augmented and alternative activation attenuated in macrophages from *MKP-1*-deficient mice. Thus, MKP-1 appears to "shift" macrophage phenotype from the proinflammatory classical activation towards the anti-inflammatory and healing-promoting alternative activation. Also, previous studies point to the induction of *MKP-1* as a potential anti-inflammatory regulatory mechanism when challenged with proinflammatory stimuli (Salojin et al., 2006). This was also confirmed in our study.

More interestingly, the anti-inflammatory glucocorticoid dexamethasone suppressed classical macrophage activation and enhanced alternative activation, and both effects were attenuated in cells from *MKP-1*-deficient animals. These findings together imply that macrophage MKP-1 is a significant factor limiting inflammation and promoting tissue healing. Thus, MKP-1 shows promise as an anti-inflammatory drug

target. Previously, MKP-1 expression has been shown to be increased by some anti-inflammatory drugs such as glucocorticoids (Abraham et al., 2006), the antirheumatic drug aurothiomalate (Nieminen et al., 2010) and the PDE4 inhibitor rolipram (Korhonen et al., 2013), and this is thought to at least partly mediate their anti-inflammatory effects.

In contrast to the relatively well-established suppressive effects of MKP-1 on classical macrophage activation, its effects, if any, on the alternative phenotype have been much less studied. Kim and coworkers have reported that macrophages from *MKP-1* KO mice displayed suppressed IL-4-induced alternative macrophage activation (Kim et al., 2016). Our results corroborate these findings and extend them by showing that the enhancing effect of dexamethasone on alternative activation is mediated by MKP-1.

Alternative macrophage activation promotes resolution of inflammation and tissue healing (Rigamonti et al., 2014; Sindrilaru & Scharffetter-Kochanek, 2013). Thus, it could be speculated that defective healing processes may partly underlie the more severe inflammation found in *MKP-1*-deficient animals. During infection or inflammation, MKP-1 may act to maintain the balance between acute inflammatory response and later tissue repair processes.

6.2.3 Effects of *Pinus sylvestris* knot extract and nortrachelogenin on M2 macrophage phenotype

Like excessive classical activation, excessive alternative macrophage activation can also be harmful by, for example, contributing to fibrosing processes. Thus, compounds attenuating alternative activation have therapeutic potential. In Study II, we investigated the effects of *Pinus sylvestris* knot extract and its major lignan component nortrachelogenin on IL-4+IL-13-induced ("wound-healing") alternative macrophage activation. The extract decreased the expression of markers of this phenotype in both murine and human macrophages. Nortrachelogenin had similar effects, which suggests that this compound is (at least partly) responsible for the effects of the pine knot extract. The effects of nortrachelogenin were also studied *in vivo* in bleomycin-induced murine dermal fibrosis, a disease model of scleroderma known to be associated with alternative macrophage activation. In this model, nortrachelogenin prevented the development of skin fibrosis. Reduced expression

of several collagens (as molecular markers of fibrosis) as well as arginase 1, a marker of alternative macrophage activation, were also observed.

In their native role, lignans function as a part of plant's immune systems, possessing antibacterial and antifungal properties (Välimaa et al., 2007). In mammals, they have been shown to attenuate inflammation by reducing *COX-2* expression and inhibiting NF-κB signaling (Baumgartner et al., 2011). Accordingly, they display protective effects in several disease and injury models associated with excessive inflammation, such as carrageenan-induced paw edema (Laavola et al., 2017), cerebral ischemia-reperfusion injury (Baumgartner et al., 2011) and sepsis (Li et al., 2014). Interestingly, they also seem to attenuate T_H2 cell-mediated allergic lung inflammation (Shin et al., 2013).

Lignans have been previously studied as potential therapeutics for inflammation and cancer (Rahimifard et al., 2017; Zhou et al, 2021). However, nortrachelogenin seems to be less studied than many other agents, and there are large differences between the effects of different lignans and polyphenols in general (as also seen in this thesis). The anti-inflammatory effects of nortrachelogenin have previously been shown to be mediated (at least partly) via reduced classical macrophage activation (Laavola et al., 2017), a trait shared by several lignans and related plant polyphenols (Eräsalo et al., 2018; Kim et al., 2019). However, nortrachelogenin (along with matairesinol as identified in our study) seems to be relatively unique among these compounds in that it also suppresses IL-4+IL-13 -induced alternative macrophage activation *in vitro*. Interestingly, the stilbene pinosylvin, another component of *Pinus sylvestris* knot extract, upregulated *ArgI* expression in our data. Promotion of M2 activation by pinosylvin has later been confirmed (Kivimäki et al., 2021).

Regarding the mechanisms by which nortrachelogenin inhibits alternative activation, we showed that whole pine knot extract and nortrachelogenin decrease the expression of *PPARγ*, a transcription factor that plays an important role in alternative macrophage activation (Chawla, 2010). In contrast, knot extract and nortrachelogenin did not affect phosphorylation of STAT6, another important regulator of alternative activation. This seeming discrepancy might be explained by noting that STAT6 and PPARγ seem to promote this macrophage phenotype in a synergistic but independent manner. For instance, Odegaard and coworkers have shown that IL-4-induced STAT6 phosphorylation remains unaltered in

PPARγ knock-out mice, but alternative macrophage activation is still significantly reduced in these animals (Odegaard et al., 2007).

6.2.4 Effects of nortrachelogenin on bleomycin-induced dermal fibrosis

It is extremely common for an intervention to show theoretically beneficial effects *in vitro* but fail to translate those into an effective therapeutic action *in vivo* (Mattes et al. 2020). Thus, demonstrating the effects of a potential therapeutic compound in an *in vivo* model system is an important "intermediate" step for determining its real medical worth. In Study II, we chose to test the effects of nortrachelogenin in bleomycin-induced murine dermal fibrosis. Fibrosis in general is associated with alternative macrophage activation: in addition to producing ECM precursors from ornithine via arginase (Comalada, Yeramian et al., 2012), these cells also direct the actions of other cells involved in the process, such as fibroblasts and myofibroblasts, by producing profibrotic mediators (Sindrilaru & Scharffetter-Kochanek, 2013). Increased presence of alternatively activated macrophages has been demonstrated in various fibrotic states such as dermal sclerosis, chronic pancreatitis and idiopathic pulmonary fibrosis (Maier et al., 2017; Murthy et al., 2015; Xue et al., 2015). In Study II, we found the presence of alternatively activated macrophages (as measured by increased expression of *ArgI* and several collagens) to be increased in bleomycin-induced skin fibrosis. This increase, as well as increased dermal thickness, was attenuated by nortrachelogenin, demonstrating that the compound has beneficial effects on the development of adverse fibrosing processes *in vivo*. There are currently no widely applicable effective treatments for fibrotic diseases, and compounds (such as nortrachelogenin) modulating alternative macrophage activation could be a potential approach for meeting this unmet therapeutic need. A positive aspect of nortrachelogenin's effects is that it doesn't "shift" macrophage phenotype towards the proinflammatory classical activation but rather suppresses both M1 and M2 macrophage phenotypes. Thus, potential antifibrotic future therapeutics derived from nortrachelogenin might avoid adverse effects resulting from increased M1 activation.

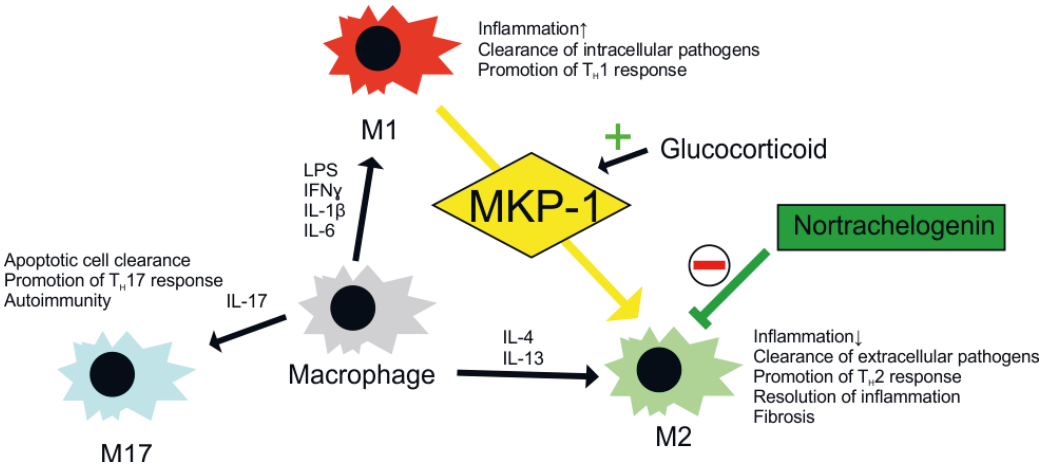

Figure 6-1.Summary of macrophage phenotypes and factors affecting them. IL = interleukin, IFNγ = interferon gamma, LPS = lipopolysaccharide, T_H = T helper cell.

6.3 Chondrocyte phenotypes in inflammation

6.3.1 Effects of the central T_H1 / T_H2 / T_H17 cytokines on OA chondrocyte phenotype

The concept of macrophage polarization was modeled after that of T_H cells, and it has proven to be an effective framework for exploring the roles of macrophages in different physiological and disease states. Chondrocytes are also characterized by great phenotypic heterogeneity, and in Study III, we sought to extend the concept of T_H cell / macrophage polarization to chondrocytes (Figure 6-2). When treated with the central T_H1/T_H2/T_H17 cytokines, OA chondrocytes were indeed found to assume distinct phenotypes. The C(IL-1β) phenotype seems to be characterized by a widespread, strong upregulation of inflammatory and catabolic factors. A smaller set of inflammatory and chemotactic factors is induced in the C(IL-17) phenotype. The C(IFNγ) phenotype appears quite distinct from those induced by both IL-1β and IL-17, with strong upregulation of several genes involved in antigen processing and presentation. Chondrocytes are not classically considered antigen-presenting cells, but some previous interesting results suggest that they present cartilage proteoglycans as antigens to CD8+ T cells, potentially contributing to joint inflammation in arthritis (Kuhne et al., 2009; Sengprasert et al., 2022). Effects of the

anti-inflammatory T_H2 cytokine IL-4 on OA chondrocyte phenotype were much more modest. Some inflammation-regulating genes were upregulated in C(IL-4) cells, while most of the downregulated genes were associated with cell proliferation and migration as well as TGFβ signaling.

When the functional gene categories affected by the different cytokines were studied using the GO database, all three proinflammatory cytokines affected several terms associated with inflammation. The C(IL-1β) phenotype, with its long lists of markedly up- and downregulated genes, was alone characterized by several GO terms such as ECM metabolism and collagen catabolism as well as cell adhesion and migration. Several terms associated with antigen processing and presentation were significantly affected in C(IFNγ) cells alone.

This thesis is the first to characterize the chondrocyte phenotypes induced by central $T_H1/T_H2/T_H17$ cytokines. These phenotypes can be thought to be at least partly analogous to the T_H cell and macrophage phenotypes mentioned before (see pp. 24 and 25); C(IL-1β) cells have heavily proinflammatory characteristics (analogous to T_H1/M1), C(IL-17) is characterized by the induction of a more limited set of inflammatory mediators (T_H17) and C(IL-4) promotes regulation of inflammation (T_H2). The potential antigen-presenting functions of C(IFNγ) warrant further studies in the setting of arthritis.

6.3.2 Effects of dexamethasone on OA chondrocyte phenotype

Medications used in the treatment of OA are known to affect gene expression in chondrocytes, potentially altering the phenotype of these cells into either beneficial or harmful directions (Cooke et al. 2020). Among the most widely used and (generally) the most potent of these medications are glucocorticoids, compounds with well-known metabolic and anti-inflammatory effects.

In Study IV, the effects of the GC dexamethasone on chondrocyte phenotype were studied in cells from OA patients (Figure 6-2). RNA-Seq provides a comprehensive view of gene expression in a tissue, and this information can be complemented by confirming the results with another method and studying differentially expressed genes "holistically" with functional analysis tools. To our knowledge, no such comprehensive sequencing studies on the effects of GCs on OA chondrocytes have been performed previously.

One group of genes markedly affected by dexamethasone was those associated with inflammation. Constant low-grade inflammation, along with transient inflammatory exacerbations, is a central feature of OA, and GCs are known to have profound anti-inflammatory and analgesic effects. However, there has previously been little information about their comprehensive effects on chondrocyte gene expression. In our data, dexamethasone markedly affected the expression of a large number of genes: a total of 1235 genes were up- or downregulated with a fold change (FC) > 2.0 in either direction. Among the genes downregulated by dexamethasone were several well-known inflammatory factors such as *COX-2*, *IL6* and *CCL2/MCP-1* (Raghu et al., 2017; Wojdasiewicz et al., 2014). Dexamethasone also downregulated the expression of many ECM-degrading enzymes such as *MMP*s *1*, *13* and *16*, while various collagens and anabolic factors were also downregulated.

Other intriguing effects of dexamethasone include the upregulation of *FOXO3* and *SOD2*, potentially indicating activation of antioxidative and cytoprotective signaling pathways (Lane et al., 2015; Shen et al., 2015). On the other hand, dexamethasone strongly enhanced the expression of *KLF9*, which has been shown to sensitize cells to oxidative stress (Zucker et al., 2014) and may contribute to the previously reported increase of cellular oxidation markers by dexamethasone (Suntiparpluacha et al., 2016). Another potentially very relevant effect is the dexamethasone-induced downregulation of *NGF*, a central mediator of OA pain (Perrot, 2015). Antibodies targeting NGF are effective in the treatment of OA pain, but may also accelerate cartilage degradation in a subset of patients (Miller et al., 2017). The mechanisms of NGF blocker -induced joint degradation are still largely unknown. Increased use of the OA-affected joint, enabled by analgesia, has been hypothesized to explain the findings. However, NGF blockers also seem to accelerate the destruction of non-OA-affected (and initially painless) joints, casting doubt on this hypothesis (Miller et al., 2017). Whether GC-induced downregulation of *NGF* might have similar deleterious effects remains to be studied.

In addition to *NGF*, dexamethasone also downregulated *COX-2* and *VEGFA*. The prostaglandin products of COX-2 (particularly PGE_2) as well as VEGFA, are also involved in mediating OA pain (Hamilton et al., 2016; Mastbergen et al., 2005). Thus, their downregulation by dexamethasone is likely to contribute to the analgesic properties of GCs in OA.

Systemic GC therapy is known to have widespread effects on metabolism. They increase blood glucose and inhibit its intake into muscle and adipose tissue, increase fat breakdown and increase serum sodium concentration while decreasing that of potassium and calcium (Ritter et al., 2018b). Some of these changes are shared by metabolic syndrome, a cluster of metabolic derangements that is also a widely known risk factor for OA (Liu et al., 2020). In OA chondrocytes, dexamethasone significantly affected the expression of several genes involved in carbohydrate and lipid metabolism. One of the most strongly upregulated genes was *PDK4*, which inactivates pyruvate dehydrogenase and prevents pyruvate produced in glycolysis from progressing to oxidative phosphorylation (Kolobova et al., 2001). This indicates that GCs may shift carbohydrate metabolism from mitochondrial respiration towards glycolysis. Several genes promoting lipid synthesis and transport, such as perilipin 2 (*PLIN2*) (Conte et al., 2016) and 5'-AMP-activated protein kinase subunit gamma-2 (*PRKAG2*) (Willows et al., 2017), were also upregulated. Apolipoprotein D (*APOD*) and sestrin 3 (*SESN3*) are two genes involved in lipid metabolism whose expression has previously been shown to be reduced in OA (Shen et al., 2017; Tew et al., 2007). Both were upregulated by dexamethasone in Study IV suggesting that GCs can (at least to some extent) "normalize" OA chondrocyte metabolism.

6.3.3 Effects of ibuprofen on OA chondrocyte phenotype

Along with glucocorticoids, nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in arthritis. They reduce inflammation and pain by inhibiting prostanoid, particularly PGE_2, synthesis by COX enzymes. COX-2 activity and PGE_2 production has previously been shown to be increased in OA chondrocytes, and PGE_2 has been proposed to modulate cartilage proteoglycan degradation (Hardy et al., 2002). These findings suggest that inhibiting this process might "normalize" the chondrocyte phenotype. Accordingly, the NSAID ibuprofen has been reported to decrease markers of joint tissue turnover in knee OA (Gineyts et al., 2004).

In contrast to the aforementioned potential beneficial effects, some studies have implicated NSAIDs in accelerating radiological OA progression. There is, however, considerable controversy over these results, and the studies have been criticized for not controlling for the severity of joint pain as a potential confounder (Ding, 2006). To our knowledge, comprehensive effects of NSAIDs on chondrocyte gene expression have not been studied previously.

In our data (Study V), ibuprofen alone (in the absence of added cytokines) had no significant effect on the expression of any gene in OA chondrocytes (Figure 6-2). This is somewhat unexpected, as even "naive" human chondrocytes express ibuprofen's main target COX-2 at a relatively high level and have been shown to produce PGE_2 (Brochhausen et al., 2006). This lack of observed effects by ibuprofen probably reflects the effect of multiple comparison correction (FDR) in RNA-Seq results. Generally, ibuprofen had somewhat more modest effects (as measured by FC values) than dexamethasone in Study IV (even in IL-1β-treated cells). In the absence of external stimulus, the relatively steep statistical adjustment performed when analyzing the tens of thousands of genes identified by RNA-Seq "dilutes" those fold changes to statistical insignificance. Even though it is possible (or indeed probable) that minor changes in gene expression by ibuprofen could be detected in chondrocytes when using less stringent multiple comparison procedures, the present results reinforce the belief that ibuprofen has no overt deleterious effects on chondrocyte gene expression.

In inflammatory conditions (when administered to chondrocytes exposed to the central proinflammatory cytokine IL-1β to mimic the low-grade inflammation present in OA cartilage), the effects of ibuprofen on chondrocyte phenotype were more interesting. In this context, ibuprofen increased the expression of several anti-inflammatory genes. On the other hand, some proinflammatory factors were among the upregulated genes.

Interleukins 6 and 23 are examples of proinflammatory cytokines downregulated by ibuprofen, with IL-23 showing especially strong downregulation. The potential local cartilage effects of IL23 in OA appear relatively understudied, but its serum levels in patients have been found to be higher compared to controls (Askari et al., 2016). In rheumatoid arthritis, IL-23 shifts T_H cells towards the T_H17 phenotype and increases IL-17 production (Yuan et al., 2019). The monoclonal IL-23 antibody ustekinumab is approved for use in seronegative arthritides, with other agents being investigated (Ceribelli et al., 2021).

Intriguing examples of genes upregulated by ibuprofen include peroxisome proliferator activated receptor gamma (*PPARG*) and its coactivator 1 beta (*PPARGC1B*). *PPARG* has been shown to be downregulated in OA cartilage (Afif et al., 2007), and its presence may affect OA pathogenesis by suppressing joint inflammation, reducing the production of catabolic enzymes and inhibiting

chondrocyte apoptosis (Fahmi et al., 2011). On the other hand, induction of granulocyte-macrophage colony stimulating factor (*CSF2*/*GM-CSF*) by ibuprofen can be regarded as a potentially harmful effect as this factor has been shown to promote OA development and pain (Conaghan et al., 2019). To our knowledge, this is the first study linking NSAIDs to GM-CSF production in chondrocytes.

When the functions of the differentially expressed genes were studied with IPA (canonical pathway analysis), integrin signaling was most strongly activated. This is intriguing as dysregulated integrin signaling may play a role in the pathogenesis of OA (Peters et al., 2002). Another interesting finding is the activation of MAPK/ERK signaling, as some of the potentially beneficial effects of ibuprofen on chondrocytes (reduction of apoptosis and prevention of dedifferentiation) may be independent of PGE_2 production and be instead at least partly mediated via ERK (Yoon et al., 2003). cAMP signaling was also activated by ibuprofen, and this signaling pathway has previously been identified as a central factor affecting chondrocyte differentiation (Carroll & Ravid, 2013).

Phosphatase and tensin homolog (PTEN) signaling was the only IPA canonical pathway significantly inhibited by ibuprofen. PTEN is a modulator of phosphoinositide 3-kinase / Akt (PI3K/Akt) signaling with various potential effects including promotion of apoptosis, regulation of cell adhesion and inhibition of cell proliferation. Previously studies have shown *PTEN* to be upregulated in OA chondrocytes, where it inhibits ECM production (Iwasa et al., 2014). Blocking PTEN signaling may slow the development of osteoarthritic changes in cartilage (Chen et al., 2020), suggesting another potentially beneficial action of ibuprofen on OA pathogenesis. To our knowledge, PTEN hasn't previously been linked to NSAIDs in cartilage.

Interestingly, genes downregulated by ibuprofen include some whose expression has previously been shown to be increased in inflamed OA synovium compared to non-inflamed, namely hyaluronan synthase 1 (*HAS1*) and stanniocalcin 1 (*STC1*) (Lambert et al., 2014). This suggests that NSAIDs can, to some extent, "normalize" the phenotype of OA joint tissue under inflammatory conditions. HAS1 is a central enzyme participating in the synthesis of hyaluronan, an important component of cartilage ECM and synovial fluid. It also has proinflammatory properties mediated via the production of monocyte-attracting hyaluronan coats. This suggests that in addition to COX inhibition, NSAIDs might have another beneficial anti-

inflammatory property in this setting (Jokela et al., 2015). STC1 is a protein involved in calcium and phosphate metabolism, and its effects on cartilage appear to be complex. On the one hand, it appears to have an inhibitory effect on cartilage development (Wu et al., 2006). However, its expression in synovial cells has also been linked to slower OA progression (Wu et al., 2019).

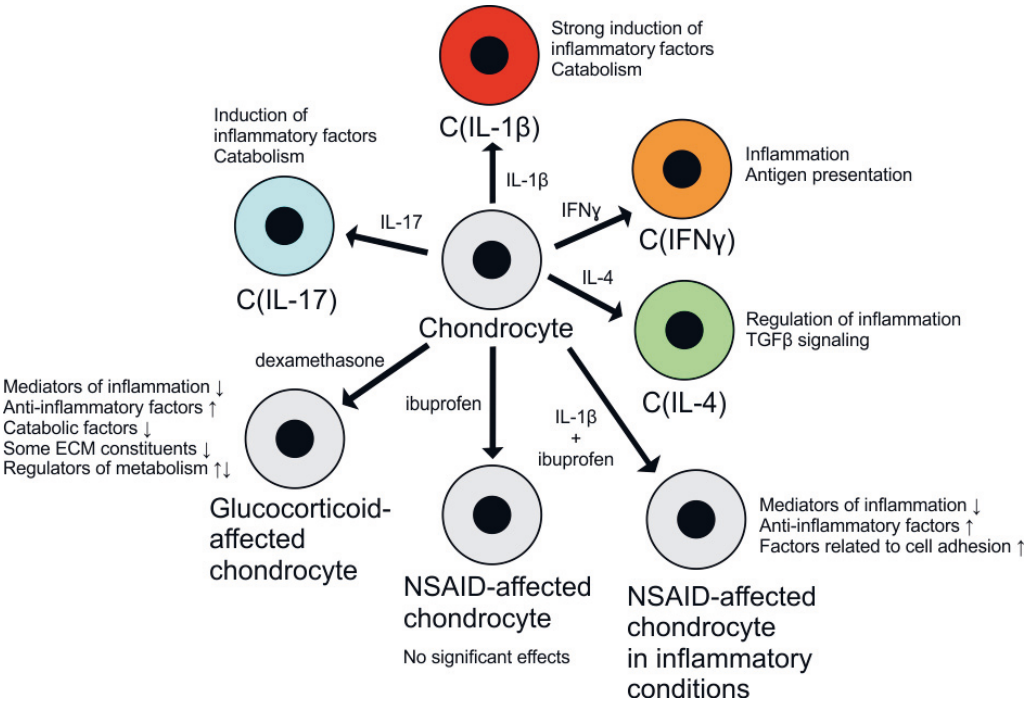

Figure 6-2.Summary of chondrocyte phenotypes and factors affecting them. IL = interleukin, IFNγ = interferon gamma, NSAID = nonsteroidal anti-inflmmatory drug, ECM = extracellular matrix, C(IL-17) = chondrocyte phenotype induced by interleukin 17, etc.

6.4 Study limitations

The present results have potential "general" limitations affecting their interpretation and future translation into clinical care. One factor related to Studies I and II is that immune response differs between the human and the mouse. For example, mice are much less sensitive to the effects of TLR agonists (such as LPS) compared with humans and require doses that are several orders of magnitude higher to trigger strong inflammatory responses (Zschaler et al., 2014). Also, the differences in

arginine metabolism (see p. 35) and M1/M2 markers (see p. 37) between human and murine macrophages should be considered when interpreting data obtained from murine macrophages. Human macrophages were used in conjunction with murine cells in Study II to address this issue. Also, the chondrocytes used in Studies III-V were exclusively of human (OA patient) origin.

Another potential question concerning the generalizability of the results of Study II in the setting of states such as arthrofibrosis is the degree of similarity between fibrotic states in dermis (as in the scleroderma model used in Study II) and synovium (as in arthrofibrosis). Dermis and synovium are histologically different; dermis consists of a deeper layer of dense irregular connective tissue and a superficial papillar layer composed of looser collagen fibers, while the synovial membrane is composed of a dense subintimal layer and the intima containing type A and B synoviocytes (Scanzello et al., 2021). Despite this, both fibroblasts and macrophages (or cells heavily resembling them) are present in both tissues, and relatively similar inflammatory reactions can be observed in both. One example of this is psoriasis, a disease that commonly manifests as both dermal inflammation and arthritis (Belasco et al., 2015).

Interestingly, synovial fibrosis has recently also been implicated as a central feature of osteoarthritis (Zhang et al., 2021). The pathophysiology of synovial fibrosis, and its relation to arthritis, is not understood in detail. Interestingly, there are hints that synovial fibrosis may involve excessive activation of inflammation-resolving and tissue-healing processes corresponding to alternative macrophage activation; accordingly, M2-associated factors such as TGFβ are implicated in synovial fibrosis (Remst et al., 2015).

The chondrocyte isolation method used in the study harvests all of the chondrocyte present in the cartilage sample, including those from all of the different cartilage layers (see p. 40). There may be differences in gene expression between these chondrocyte populations (Mobasheri et al., 2021), and sequencing them simultaneously can be expected to "average out" these differences. On the other hand, this can be thought to resemble the clinical situation, in which a medication (at least theoretically) affects the gene expression of all articular cells, and the "net sum" of these changes determines the clinical effect(s) of the medication. In the future, methods such as single-cell RNA-Seq would allow for a more accurate characterization of different chondrocyte populations in the cartilage.

6.5 Clinical implications

This thesis established for the first time that the phenotype of macrophages, cells acting as "master regulators" of inflammation, can be modulated by the endogenous phosphatase MKP-1 as well as exogenously by nortrachelogenin, a plant polyphenol. Our research group has previously shown that anti-inflammatory medications aurothiomalate, glucocorticoids, PDE4 inhibitors and β2 agonists increase MKP-1 expression (in addition to their more familiar modes of action) (Keränen et al., 2016; Korhonen et al., 2013; Nieminen et al., 2010; Tuure et al., 2017), and thus, their effects might be mediated both via inhibition of proinflammatory classical macrophage activation and enhancement of anti-inflammatory alternative activation. This gives important insights into the use of these medications and could inform the design of potential more selective MKP-1-targeted therapies in the future. As macrophages direct the functions of many cell types (Abbas et al., 2018c), compounds that selectively activate MKP-1 or increase its expression could function as widely effective anti-inflammatory medications with less side effects than, for example, GCs.

On the other hand, compounds derived from nortrachelogenin might be used to inhibit the excessive alternative macrophage activation observed in fibrosing diseases, a group of pathological states for which there is currently a dearth of effective treatments available. Currently approved medications for diseases such as pulmonary fibrosis (pirfenidone and nintedanib) are thought to work primarily via inhibition of fibroblast function (along with possible anti-inflammatory effects) (Raghu et al., 2015). Thus, compounds targeting macrophages might offer an interesting alternative or complementary therapeutic avenue.

In addition, it was shown for the first time that chondrocytes, cells of the cartilage and major actors in osteoarthritis, can adopt distinct phenotypes in response to the central cytokines associated with different T_H cell phenotypes. This increases our understanding of OA pathophysiology, and may inform future therapies that could change chondrocyte phenotype into a "healthier" direction. The concept of polarized chondrocyte phenotypes, and potential therapies derived from it, could also likely be extended to other forms of arthritis. As the major inflammatory cytokines (especially IL-1β and IL-17, as well as to lesser extent IFNγ) seem to change chondrocyte phenotype towards degraded cartilage (as determined by

comparing our results to those of Coutinho de Almeida et al., 2019), counteracting the effects of those cytokines could be a promising therapeutic strategy.

The two last parts of this thesis investigated the effects of GCs and NSAIDs on chondrocyte phenotype. To our knowledge, no such comprehensive genome-wide analyses of the effects of GCs or NSAID on chondrocyte phenotype have been previously performed. Both of these classes of drugs are used in the treatment of OA, but their detailed benefit/harm ratios regarding cartilage are unknown. The results of this thesis show that both GCs and NSAIDs (dexamethasone and ibuprofen, respectively) induce the expression of anti-inflammatory genes in cartilage, which can be regarded as a beneficial effect in the context of inflammatory OA. Dexamethasone also downregulated central mediators of OA pain, which reinforces the notion of intra-articular GCs having analgesic effects in OA. On the other hand, dexamethasone downregulated the expression of many collagens and other ECM constituents (along with a very large number of other genes), suggesting that it might impair cartilage regeneration. As clinical studies have not displayed intra-articular GCs having clear deleterious effects on cartilage volume or structure (McAlindon et al., 2017; Raynauld et al., 2003; Spitzer et al., 2019), these potentially deleterious gene expression changes should probably not cause great alarm by themselves. However, more detailed studies investigating the protein-level effects of GCs on the production of ECM components would shed light on this issue.

In the absence of external cytokines, no significant changes in gene expression by ibuprofen were observed. In OA, ibuprofen and other NSAIDs are generally used perorally or topically. After oral administration, they diffuse into synovial fluid, and the observed synovial fluid concentrations are generally of comparable magnitude to those in plasma (Netter et al., 1989). From synovial fluid, they further diffuse into cartilage and can thus affect chondrocytes (Gaucher et al., 1983). The fact that even direct treatment with ibuprofen had no significant effects on the transcriptome of "naïve" OA chondrocytes strengthens the assumption that NSAIDs are generally safe for cartilage.

An aspect that affects the use of NSAIDs and GCs is the relative difference between the time courses of their effects. NSAIDs act relatively quickly by inhibiting prostaglandin synthesis via the COX enzymes; reduced prostaglandin levels then have direct anti-inflammatory and analgesic effects. They may also affect gene expression by altering intracellular cAMP levels and the activity of the CREB

transcription factor. However, as evidenced by Study V, in which ibuprofen had no significant effects on chondrocyte phenotype in the absence of proinflammatory stimuli, these effects can be expected to be rather modest in chondrocytes. On the other hand, the main mechanisms of action of GCs is that they affect gene expression via GC-glucocorticoid receptor complexes (Leppänen & Moilanen, 2018; Wang, P. et al., 2010). NSAIDs are generally taken perorally several times per day (1-4 times per day depending on the compound), for several days or weeks at a time. As they are relatively effective and available over the counter, long-term or continuous NSAID use is also quite common (though not generally recommended due to the risks of gastrointestinal and cardiovascular adverse events) (Ritter et al., 2018a). In contrast, intra-articular GCs are given by injection during inflammatory exacerbations, and an interval of several months between repeated injections is generally recommended (Stephens et al., 2008). This would suggest that NSAIDs exert effects on cartilage for longer periods of time (and therefore might have greater total impact) than GCs. However, at least two factors complicate this picture. First, long-acting glucocorticoids are often used in intra-articular injections: for example, triamcinolone acetonide can persist in the joint for several weeks after the injection, and special long-acting formulations can persist for months (Kraus et al., 2018). Second, the multifaceted changes in chondrocyte gene expression caused by GCs (as evidenced in Study IV) can persist, and cause diverse second-order effects, long after the initial GC stimulus has faded.

6.6 Further directions

The results of this thesis could inform further studies into several different directions.

In this thesis, MKP-1 was shown to inhibit classical and to promote alternative macrophage activation. In addition, MKP-1 has previously been shown to be a central regulator of macrophage metabolism, a factor that is also intimately linked to macrophage phenotype (Kim et al., 2016). Thus, investigating the metabolic effects of MKP-1 in macrophages is an interesting avenue for future research. Also, the T_H17-associated M17 macrophage phenotype is relatively understudied and less well characterized than M1 and M2, and interestingly, MKP-1 has been shown to affect IL-17 signaling (Huang et al., 2015). Therefore, it would be intriguing to study the potential effects of MKP-1 deficiency on M17 activation. Also, it would be

interesting to study the role(s) of MKP-1 in the cytokine-induced chondrocyte phenotypes identified in Study III and in mediating the effects of dexamethasone on chondrocyte phenotype (Study IV).

Nortrachelogenin was found to inhibit alternative macrophage activation and to attenuate bleomycin-induced dermal fibrosis in mice. In order to develop antifibrotic medications from nortrachelogenin (or its derivatives), it would be important to further elucidate its effects. This could involve measuring a wide array of alternative (as well as classical) macrophage activation markers in fibrotic skin (perhaps using immunohistochemistry or related methods). Low bioavailability is a problem with many bioactive polyphenols that have been studied as potential medications (Di Lorenzo et al., 2021). Nortrachelogenin has been shown to be absorbed in an active form following both peroral and intraperitoneal administration (Sachin et al., 2008; Yatkin et al., 2014); however, its bioavailability might be further enhanced by developing more lipophilic derivatives or liposomal/micellar formulations, approaches that have shown efficacy with other lignans (Wang et al., 2011).

The chondrocyte phenotypes identified in Study III could be further characterized and studied on a functional level by identifying robust molecular/functional markers of these phenotypes and measuring them in the protein level. Some interesting functional details were observed in our data: for example, IFNγ activated pathways related to antigen processing and presentation, and the potential role of these pathways in arthritis warrants further study. IL-4 was found to downregulate genes involved in TGFβ signaling, a pathway that has been linked with OA pathogenesis (Zhen et al., 2013). Thus, it would be interesting to study the potential OA-attenuating effects of IL-4 on a functional level.

The effects of dexamethasone on chondrocyte phenotype could be further elucidated by studying the relevant functions (like ECM component production and metabolic changes) in protein level. Also, the (patho)physiological implications of certain more specific observed effects of dexamethasone, such as downregulation of NGF and VEGF, could be further elucidated by treating chondrocytes with these growth factors or their inhibitors. As GCs have widespread effects on metabolism, their functions in the context of so-called metabolic phenotype of OA would be an interesting potential avenue of further study. Also, it would be intriguing to investigate the effects of dexamethasone on the chondrocyte phenotypes identified in Study IV.

The effects of ibuprofen on cartilage could be further elucidated by modulating the functional pathways identified in this thesis, such as integrin signaling (activated by ibuprofen) and PTEN signaling (inhibited by ibuprofen) and observing the effects of these interventions on chondrocyte function and metabolism could shed further light on their potential roles in OA. Also, it would be interesting to compare the effects of a nonselective NSAID such as ibuprofen with a COX-2 selective agent to try to differentiate the roles of the two COX isoforms in mediating the effects of ibuprofen. The effects of ibuprofen on other chondrocytes characterized in Study IV [apart from C(IL-1β)] would also be intriguing to explore.

On a more general level, obtaining chondrocytes from patients with different putative OA phenotypes (metabolic, posttraumatic etc.) would be a very interesting avenue of future research; it would facilitate the study of different chondrocyte phenotypes in these patients and allow us to see whether these phenotypes might correlate to clinical OA phenotypes.

SUMMARY AND CONCLUSIONS

The major findings and conclusions are as follows:

I) MKP-1 shifts macrophage phenotype away from the proinflammatory classical activation and towards the anti-inflammatory alternative activation. MKP-1 also partly mediates the effects of glucocorticoids that suppress classical and promote alternative macrophage activation. This suggests that MKP-1 is a central regulator of inflammation not solely by suppressing proinflammatory cellular responses, but also by promoting anti-inflammatory ones. Thus, compounds that increase the expression and/or activity of MKP-1 display promise as potential therapeutics for inflammatory diseases.

II) Nortrachelogenin, the main lignan component of *Pinus sylvestris* knotwood extract, attenuates alternative macrophage activation. *In vivo,* it ameliorates bleomycin-induced murine dermal fibrosis, a condition that resembles scleroderma in humans and is associated with excessive alternative macrophage activation. Thus, nortrachelogenin or compounds derived from it could show promise as disease-modifying treatments of fibrosing diseases.

III) The major $T_H1/T_H2/T_H17$ cytokines IFNγ, IL-1β, IL-4 and IL-17 are able to induce distinct phenotypes in OA chondrocytes. Of these, the C(IL-1β) phenotype is characterized by widespread, strong proinflammatory and procatabolic activation, the C(IFNγ) phenotype by antigen processing and presentation, the C(IL-4) phenotype by regulation of inflammation and the C(IL-17) phenotype by somewhat more limited proinflammatory action [compared to C(IL-1β)]. These results suggest that modulating the phenotype of chondrocytes might be a promising therapeutic strategy for affecting the course and symptoms of OA.

IV) The glucocorticoid dexamethasone has a widespread effect on the phenotype of OA chondrocytes, with over 1000 genes being up- or downregulated with a fold change (FC) ≥ 2.0 in either direction. This included a downregulation of several inflammatory factors and upregulation of anti-inflammatory genes, while many catabolic factors were downregulated by dexamethasone. Widespread effects on genes regulating lipid and carbohydrate metabolism were also observed. When the results were compared with previous genome-wide expression analyses studying chondrocyte transcriptomes in degraded and preserved OA cartilage, dexamethasone was found to partly shift chondrocyte gene expression towards the latter. These results confirm that GCs have multiple forms of disease-modifying potential in OA and partly normalize chondrocyte phenotype towards that of healthy cartilage.

V) The nonsteroidal anti-inflammatory drug (NSAID) ibuprofen alone doesn't significantly affect gene expression in OA chondrocytes when adjusted for multiple testing. In inflammatory conditions (in the presence of the OA-associated cytokine IL-1β), ibuprofen upregulated anti-inflammatory genes such as *PPARG* and *IL10RA*, while inflammatory factors such as *IL23A* and *IL6* were downregulated. Functional analysis revealed activation of several inflammation-regulating pathways, while PTEN signaling was inhibited. This supports the notion that in addition to their analgesic effects, NSAIDs have disease-modifying potential in OA, while no overt deleterious effects were observed.

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ORIGINAL COMMUNICATIONS

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MKP-1 promotes anti-inflammatory M(IL-4/IL-13) macrophage phenotype and mediates the anti-inflammatory effects of glucocorticoids

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MKP-1 Promotes Anti-inflammatory M(IL-4/IL-13) Macrophage Phenotype and Mediates the Anti-inflammatory Effects of Glucocorticoids

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Abstract

Macrophage polarization refers to the ability of these cells to adopt different functional phenotypes according to their environment. Mitogen-activated protein kinase phosphatase-1 (MKP-1) is known to regulate the classical lipopolysaccharide (LPS) -induced pro-inflammatory macrophage activation and the inflammatory response. Here, we investigated the effects of MKP-1 on the anti-inflammatory and healing-promoting macrophage phenotype induced by cytokines IL-4 and IL-13 and examined the potential mediator role of MKP-1 in glucocorticoid effects on the two macrophage phenotypes.

In MKP-1-deficient macrophages treated with IL-4 and IL-13 to induce the anti-inflammatory phenotype, the expression of phenotypic markers arginase 1, Ym-1 and FGF2 was reduced as compared to wild-type cells. In contrast, LPS-induced expression of the pro-inflammatory factors IL-6 and iNOS was significantly higher in MKP-1-deficient macrophages. Dexamethasone suppressed the pro-inflammatory phenotype and enhanced the anti-inflammatory phenotype. Interestingly, both of these glucocorticoid effects were attenuated in macrophages from MKP-1-deficient mice. Accordingly, dexamethasone increased MKP-1 expression in both LPS- and IL4+13-treated wild-type cells.

In conclusion, the findings support MKP-1 as an endogenous mechanism able to shift macrophage activation from the classical pro-inflammatory state towards the anti-inflammatory and healing-promoting phenotype. In addition, MKP-1 was found to mediate the anti-inflammatory effects of dexamethasone in a dualistic manner: by suppressing the pro-inflammatory macrophage activation and by enhancing the healing-promoting macrophage phenotype.

Introduction

Macrophages are important immune effector cells responsible for several physiological functions related to homeostasis and immunity [1]. They clear cellular debris and participate in recycling of nutrients (e.g. iron) [2,3]. In infections, macrophages phagocytize pathogens and present antigens to lymphocytes [4]. They also regulate inflammation by secreting pro-inflammatory and anti-inflammatory cytokines and other soluble factors. It is nowadays accepted that the macrophages performing different tasks actually display distinct phenotypes [5,6], a phenomenon known as macrophage polarization.

Macrophage phenotypes were initially modelled after the Th1/Th2 paradigm for T lymphocytes. This scheme has “classically activated” or M1 macrophages, induced by microbial products and pro-inflammatory stimuli, on one end. On the other end are macrophages that do not promote immune responses but support resolution of inflammation and tissue healing. These cells are collectively called “alternatively activated” or M2 macrophages and are often subdivided according to the phenotype-inducing stimulus and / or supposed function related to the phenotype. More recently, it has been increasingly recognized that grouping such diverse cells into two pre-determined categories is not optimal;

the characteristic set of features caused by each "polarizing" factor has been recommended to be considered as a distinct phenotype, especially concerning alternatively activated macrophages [7].

The macrophage phenotype induced by activators of toll-like receptor 4 (TLR4), such as bacterial lipopolysaccharide (LPS), along with that induced by the pro-inflammatory cytokine interferon gamma (INFγ), is a well-characterized form of "classical macrophage activation". These cells promote inflammation and effectively destroy intracellular pathogens in a setting of infection [8]. They express inducible nitric oxide synthase (iNOS), which converts L-arginine into L-citrulline simultaneously releasing nitric oxide (NO), a potent vasodilator and cytotoxic effector molecule. Other characteristic inflammatory factors produced by classically activated macrophages include interleukin 6 (IL-6) and tumour necrosis factor (TNF) [9]. While these cells play a critical role in controlling infection, excessive or dysregulated classical activation has been implicated in the development of autoimmune and degenerative diseases [2] as well as atherosclerosis [10], obesity and insulin resistance [11].

A relatively well-established example of "alternative macrophage activation" is the phenotype induced by the major Th2 cytokines IL-4 and IL-13. These IL-4 / IL-13-activated macrophages are associated with phagocytosis of tissue debris, as well as with the resolution of inflammation and wound healing [12], and are characterized by up-regulation of factors involved in fibroblast activation and extracellular matrix synthesis, such as Ym-1, arginase 1 and fibroblast growth factor 2 (FGF2) [13,14]. While pro-inflammatory macrophages produce NO from arginine, macrophages activated by IL-4 and IL-13 express arginase 1 at high levels. This enzyme converts L-arginine into L-ornithine, which in turn acts as a precursor of many extracellular matrix components supporting wound healing [15,16].

Macrophage activation by IL-4 and IL-13 is critical in the resolution of inflammation and wound healing, but excessive and / or dysregulated forms of it have been linked to aberrant fibroblast activation and pathogenesis of fibrotic diseases. Accordingly, macrophages displaying an alternative phenotype stimulated by IL-4 and IL-13 have been detected in fibrotic lungs [17], kidneys [18] and liver [15].

Mitogen-activated protein kinase phosphatases (MKPs), also known as dual specificity phosphatases (DUSPs), are enzymes that dephosphorylate mitogen-activated protein kinases (MAKPs) and thus inactivate signalling cascades mediated through these pathways [19–21]. MAPKs regulate the activity of several enzymes and transcription factors involved in inflammatory responses [22,23]. MKP-1, which preferentially dephosphorylates p38 and JNK, is the best known MAPK phosphatase [20,21,24]. MKP-1 expression is up-regulated in response to many extracellular signals and cellular stress, such as LPS, cytokines and heat shock [23,25]. MKP-1 deficiency has been associated with an enhanced inflammatory response, and increased expression of pro-inflammatory genes has been observed both in macrophages isolated from MKP-1 knockout mice and in cells treated with MKP-1 targeting siRNAs [26–29]. Accordingly, MKP-1-deficient mice develop more severe disease in models of septic shock or collagen-induced arthritis [30,31]. Intriguingly, MKP-1 expression has been shown to be increased by certain anti-inflammatory drugs, i.e. glucocorticoids [32–34], the anti-rheumatic drug aurothiomalate [35] and the PDE4 inhibitor rolipram [36], and this property is thought to mediate many of their anti-inflammatory effects.

In contrast to the relatively well-established role of MKP-1 in classical macrophage activation, its effects on alternative macrophage activation remain mostly unexplored. The aim of the present study was to investigate the regulatory role of MKP-1 in IL-4 and IL-13-

induced alternative macrophage activation in comparison to its effects on TLR4 (LPS)-induced classical macrophage activation, and to evaluate if MKP-1 has a role in mediating the anti-inflammatory effects of the glucocorticoid dexamethasone in macrophages. In the nomenclature, we adhere to the recent consensus paper [7] and refer to TLR4 activator-induced classically activated pro-inflammatory macrophage phenotype as M(LPS) cells and to IL-4 and IL-13-induced alternatively activated anti-inflammatory and healing-promoting phenotype as M(IL-4/IL-13) cells.

Materials and methods

Cell cultures and macrophage polarization

Murine J774 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co., Carlsbad, CA, USA) and harvested with trypsin-EDTA (Invitrogen Co.). Cells were cultured in 24-well plates for RNA extraction, ELISA measurements and preparation of whole-cell lysates for western blotting. Confluent cultures were exposed to fresh culture medium containing the compounds of interest.

Isolation and culturing of mouse peritoneal macrophages were carried out as described earlier [36]. In brief, male C57BL/6 MKP-1(–/–) mice (originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute [37]) were bred in the University of Tampere School of Medicine animal facilities. These mice, along with their

wild-type (WT) controls, were housed under standard conditions of light (12-12-hr light/dark cycle), temperature (22 ± 1°C) and humidity (50–60%). Food and water were provided *ad libitum*. In each experiment, cells from six WT and six knockout (KO) mice were pooled to give n=4. The experiments were repeated two to three times with similar results.

In collection of peritoneal macrophages, the experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by The National Animal Experiment Board. Mice were killed by suffocation with CO_2, followed by an immediate cervical dislocation. Primary peritoneal macrophages were obtained by intraperitoneal lavage with sterile phosphate-buffered saline (PBS) supplemented with 0.2 mM EDTA. Cells were washed, resuspended in RPMI 1640 medium supplemented with 2% heat-inactivated foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, and seeded on 24-well plates. The cells were incubated overnight and washed with medium to remove non-adherent cells before the experiments.

In order to study the type of TLR4 activation -induced classical macrophage activation, the cells were incubated with 10 ng/ml (J774) or 100 ng/ml (peritoneal macrophages) of a known activator of TLR4, bacterial LPS (Escherichia coli, 0111:B4, product number E-4391, Sigma) for 24 hr (J774) or 4 hr (peritoneal macrophages). This classical phenotype is henceforth called M(LPS). In experiments examining alternative activation, a combination of 10 ng/ml of IL-4 and IL-13 (both from R&D Systems, Minneapolis, MN, USA) was used to induce an alternative phenotype henceforth called M(IL-4/IL-13). When relevant, 1 μM dexamethasone was used in addition to LPS or IL-4 and IL-13. The concentrations of the chemical stimuli (LPS and IL-4 + IL-13) were chosen following our previous studies and literature, and the

time points used in the experiments were selected based on time series in J774 murine macrophage studies (Figs. 1 and 6) and on our preliminary experiments with peritoneal macrophages. The dexamethasone concentrations used in the experiments were chosen based on our previously published dose-response data [38].

Cell viability after treatment with the investigated compounds was excluded by modified XTT test (Cell Proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Preparation of protein extracts

At pre-determined time points, the culture medium was carefully removed and stored at -20°C for later analysis. The cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in cold lysis buffer containing 10 mM Tris base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside. After incubation on ice for 15 min., lysates were centrifuged (12,000 x *g*, 4°C, 10 min.), supernatants were collected and mixed 3:1 with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). The samples were stored at -20°C until analysed. An aliquot of the supernatant was used to determine protein concentration using the Coomassie blue method [39].

Western blotting

Prior to western blotting, samples were boiled for 10 min. and 20 μg of protein was loaded per lane on 10% or 12% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

After the transfer, the membrane was blocked with TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% of non-fat dry milk (arginase 1 and β-actin) or 5% bovine serum albumin (MKP-1) at room temperature for one hour and incubated with the primary antibody in the blocking solution for one hour at room temperature (arginase 1) or at 4°C overnight (MKP-1 and β-actin). The membrane was washed with TBS/T, incubated with the secondary antibody in the blocking solution at room temperature for one hour and washed. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and ImageQuant LAS 4000 mini imaging system (GE Healthcare). The chemiluminescent signal was quantified with ImageQuant TL 7.0 image analysis software.

Rabbit polyclonal arginase 1, MKP-1 and β-actin (loading control) antibodies (primary antibodies), and goat HRP-conjugated anti-rabbit polyclonal antibody (secondary antibody) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

ELISA

The concentration of IL-6 in culture media was determined by ELISA, using reagents from R&D Systems Europe Ltd., Abingdon, UK (Catalog DY406), adhering to the protocol detailed by the manufacturer. The detection limit was 0.3 pg/ml. All samples were assayed in duplicate.

Nitrite measurement with Griess method

Nitric oxide (NO) production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in the aqueous environment, into the culture medium by the Griess reaction [40]. All samples were assayed in duplicate.

RNA isolation and quantitative RT-PCR

At pre-determined time points, culture medium was removed, and total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.). Total RNA (100 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA was diluted 1:20 with RNase-free water and subjected to quantitative RT-PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Primers and probes for murine IL-6, iNOS, MKP-1, arginase 1, Ym-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1) were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number

4304449 revision C. The mRNA levels of basic fibroblast growth factor (FGF2) were determined by using TaqMan® Gene Expression Assay Mm00433287_m1 (Applied Biosystems). The PCR reaction parameters were as follows: incubation at 50°C for 2 min., incubation at 95°C for 10 min., followed by 40 cycles of denaturation at 95°C for 15 sec., and annealing and extension at 60°C for 1 min. Each sample was assayed in duplicate. The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2. For the FGF2 Gene Expression Assay, the relative mRNA levels were determined with the ΔΔCT method.

Statistics

Results are expressed as mean + standard error of mean (SEM). When indicated, statistical significance was calculated by ANOVA followed by Dunnett's or Bonferroni's multiple comparisons test where appropriate. Differences were considered significant at $p < 0.05$. For determining area under curve, trapezoidal integration was used. The statistical software GraphPad InStat3 was used for the analyses. The study was conducted in accordance with the BCPT policy for experimental and clinical studies [41].

Results

Markers of classical M(LPS) phenotype are increased while those of alternative M(IL-4/IL-13) phenotype are decreased in macrophages from MKP-1-deficient mice

The levels of interleukin 6 (IL-6) and inducible nitric oxide synthase (iNOS) expressions were investigated as typical markers of classical M(LPS) activation in J774 macrophages. LPS significantly increased the expression of both of these inflammatory factors, with mRNA levels peaking at 4-6 hr and returning to basal levels at 24 hr following exposure to LPS (figs. 1A-B). Accordingly, the levels of arginase 1 and Ym-1 were measured as typical markers of alternative M(IL-4/IL-13) activation. The combination of IL-4 and IL-13 increased the expression of arginase 1 and Ym-1 but the effect was rather slow in onset and mRNA levels continued to increase up to 12-24 hr following IL-4/IL-13 stimulation (figs. 1C-D).

To investigate the role of MKP-1 in classical M(LPS) and alternative M(IL-4/IL-13) macrophage activation, peritoneal macrophages were isolated from MKP-1-deficient and wild-type mice. After stimulation with LPS, IL-6 expression was 71% and iNOS expression 136% higher in macrophages from MKP-1-deficient mice compared to cells from wild-type mice (Fig. 2A-B). In contrast, when the cells were stimulated with the combination of IL-4 and IL-13 to induce the alternative phenotype, the expression of arginase 1, Ym-1 and FGF2 was significantly lower (by 42%, 86% and 37%, respectively) in the macrophages from MKP-1-deficient mice than in those from wild-type animals (Fig. 2C-E). These data suggest that MKP-1 is an endogenous factor regulating macrophage polarization from a pro-inflammatory phenotype towards an anti-inflammatory and tissue healing -promoting phenotype.

The effects of dexamethasone on M(LPS) and M(IL-4/IL-13) markers are attenuated in macrophages from MKP-1-deficient mice

Glucocorticoids are known to attenuate classical pro-inflammatory M(LPS) and to promote alternative inflammation-resolving M(IL-4/IL-13) macrophage activation, as also demonstrated here in J774 macrophages. When dexamethasone was added into the culture, it markedly inhibited IL-6 and iNOS mRNA expression (by 69% and 63%, respectively) and IL-6 and nitric oxide production (by 89% and 95%, respectively) in LPS-treated J774 macrophages (Fig. 3). In contrast, dexamethasone increased the expression of arginase 1 (mRNA by 85%, protein by 71%) and Ym-1 (by 1630%) in macrophages exposed to the combination of IL-4 and IL-13 (Fig. 4).

To study the role of MKP-1 in those glucocorticoid effects, we investigated the effects of dexamethasone in peritoneal macrophages from MKP-1-deficient and wild-type mice. As shown in figs. 5A and 5B, dexamethasone down-regulated IL-6 and iNOS expression in both wild-type and MKP-1-deficient peritoneal macrophages exposed to LPS, but the effect in macrophages from MKP-1-deficient mice was attenuated in a statistically significant manner. In contrast, dexamethasone enhanced the expression of the M(IL-4/IL-13) markers arginase 1, Ym-1 and FGF2 in peritoneal macrophages from wild-type animals. Intriguingly, the effect of dexamethasone on the M(IL-4/IL-13) markers was significantly attenuated in macrophages from MKP-1-deficient mice (figs. 5C-E). These data suggest that MKP-1 mediates the anti-inflammatory effects of glucocorticoids via two mechanisms: by suppressing pro-inflammatory macrophage activation and promoting anti-inflammatory macrophage phenotype.

Dexamethasone enhances MKP-1 expression in murine macrophages

To support the role of MKP-1 in the observed glucocorticoid effects, we studied the effects of dexamethasone on MKP-1 expression. In the resting state, peritoneal macrophages from wild-type mice expressed MKP-1 at low levels. When dexamethasone was introduced to the culture, it increased MKP-1 expression up to five-fold. The MKP-1 increasing effect of dexamethasone was duplicated when it was added in combination with LPS or IL-4 and IL-13 (Fig. 6A).

Similar effects were observed in J774 cells (figs. 6B-D). The time-course of MKP-1 expression in J774 macrophages (Fig. 6B) showed a rapid increase which peaked at 1 hr and was followed by another peak at 4 hr possibly induced by pro-inflammatory cytokines trigged by LPS stimulation or feedback loops between MKP-1 and p38 kinase [42,43]. Dexamethasone enhanced MKP-1 expression in unstimulated J774 cells as well as in those exposed to LPS or IL-4 + IL-13, the combination of LPS and dexamethasone being able to induce the highest increase. The effects seen at mRNA level were also translated to protein levels (Fig. 6D).

Discussion

In the present study, we show that both classical M(LPS) and alternative M(IL-4/IL-13) macrophage activation are significantly altered in cells from MKP-1-deficient mice. Opposite directions in the changes were observed: M(IL-4/IL-13) activation was attenuated whereas M(LPS) activation was augmented in macrophages from MKP-1-deficient animals. These findings display a central role of MKP-1 in the regulation of macrophage polarization towards the M(IL-4/IL-13) phenotype, which is critical in the resolution of inflammation and wound

healing. More interestingly, the anti-inflammatory steroid dexamethasone was found to suppress M(LPS) activation and to enhance M(IL-4/IL-13) activation, and both of these effects were attenuated in cells from MKP-1-deficient animals. These findings together imply that MKP-1 is a significant mechanism in limiting inflammation and promoting tissue healing, and as such, can be considered a promising anti-inflammatory drug target.

The effects of MKP-1 on pro-inflammatory states, corresponding to M(LPS) activation, have been studied previously [21–23]. MKP-1 has been shown to be up-regulated, possibly as a regulatory feedback mechanism, by pro-inflammatory stimuli such as IL-1 and bacterial LPS [26–29], and this was also confirmed in the present study. In addition, we found that the expression levels of M(LPS) markers iNOS and IL-6 were increased in macrophages from MKP-1-deficient mice as compared to cells from wild-type animals. These findings are supported by previous studies [28–30]. In animal models, MKP-1 deficiency has been shown to increase lethality in experimentally induced sepsis and endotoxic shock [30,41], as well as to cause increased inflammation and osteolysis in experimental arthritis and periodontitis [31,42]. Together these findings support the view that MKP-1 is a part of an endogenous feedback loop to control and limit inflammation mediated by pro-inflammatory classically activated macrophages [21,26].

In contrast to the well-established suppressive effects of MKP-1 on M(LPS) activation, very little is known about its possible effects on M(IL-4/IL-13) and other alternatively activated macrophage phenotypes. In connection to their studies on MKP-1 and macrophages in atherosclerosis, Kim and co-workers recently reported that MKP-1 deficiency suppressed IL-4-induced conversion of macrophages towards the M2 phenotype [43]. Our results corroborate these findings and extend them by showing that the enhancing effect of dexamethasone on M(IL-4/IL-13) activation is also mediated by MKP-1. Taken together, in

primary peritoneal macrophages from MKP-1-deficient animals, the expression of the markers of inflammation-resolving M(IL-4/IL-13) phenotype in response to the respective stimuli was significantly attenuated, along with the increased expression of markers of the pro-inflammatory M(LPS) phenotype in response to the respective factor. These findings are plausible evidence that MKP-1 regulates the balance in functional macrophage phenotypes and indicate that MKP-1 shifts macrophage polarization towards an inflammation-alleviating phenotype. MKP-1 has also been shown to regulate the metabolic aspects of macrophage phenotype [43], factors that the present study was unable to address. Investigating these factors further is a promising avenue of future research.

The alternative macrophage phenotype triggers resolution of inflammation and promotes wound healing [44–46]. Thus, one could speculate that defective activation of healing processes may be involved in the more severe inflammatory responses found in MKP-1-deficient animals in experimental models such as arthritis, inflammatory bone loss and sepsis [31,41,47]. The present results are also supported by the findings that muscle healing is impaired in MKP-1-deficient animals [48], and that MKP-1 is expressed at high levels in healing skin wounds [49]. This implies that during infection / inflammation, MKP-1 may serve as a factor maintaining balance between the acute inflammatory response and later wound healing / tissue repair processes.

MKP-1 expression is increased by glucocorticoids [33,50], which was also seen in the present study, and MKP-1 has been shown to mediate some of the anti-inflammatory effects of these compounds in macrophages and other cells exposed to pro-inflammatory stimuli [32,34]. In the present study, LPS-induced IL-6 and iNOS expression was down-regulated by dexamethasone in peritoneal macrophages from wild-type animals, but this effect was impaired in macrophages from MKP-1-deficient mice, and these results are in line with the

published data [29,51]. Dexamethasone was also found to increase the levels of markers of inflammation-resolving M(IL-4/IL-13) phenotype as shown previously [52,53]. Notably, the expression of M(IL-4/IL-13) markers induced by dexamethasone was impaired in macrophages from MKP-1-deficient mice as compared to those from wild-type animals. These data suggest that the dexamethasone-induced healing-promoting M(IL-4/IL-13) macrophage activation is at least partly mediated by MKP-1, which is a novel finding. Together, these results suggest that MKP-1 is a central endogenous mediator of the anti-inflammatory effects of glucocorticoids, both by suppressing pro-inflammatory macrophage phenotypes and by shifting macrophage activation towards anti-inflammatory and healing-promoting phenotypes. These data support the importance of MKP-1 as an anti-inflammatory drug target, together with the earlier findings showing that the effects of compounds such as the anti-rheumatic drug aurothiomalate, PDE4 inhibitor rolipram, β_2 receptor agonist salbutamol and vitamin D are at least partly mediated by MKP-1 [35,36,54,55].

In conclusion, we found that the expression of M(LPS) markers (iNOS and IL-6) was suppressed by MKP-1, while the expression of M(IL-4/IL-13) markers (arginase 1, Ym-1 and FGF2) induced by the respective cytokines was enhanced by MKP-1. This suggests that MKP-1 limits the magnitude of the acute inflammatory response and stimulates the onset of tissue repair processes following inflammation and tissue injury. Furthermore, MKP-1 seems to be an important factor mediating the anti-inflammatory actions of glucocorticoids via two mechanisms: by suppressing pro-inflammatory macrophage activation and by promoting the anti-inflammatory macrophage phenotypes that mediate healing processes.

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Figures

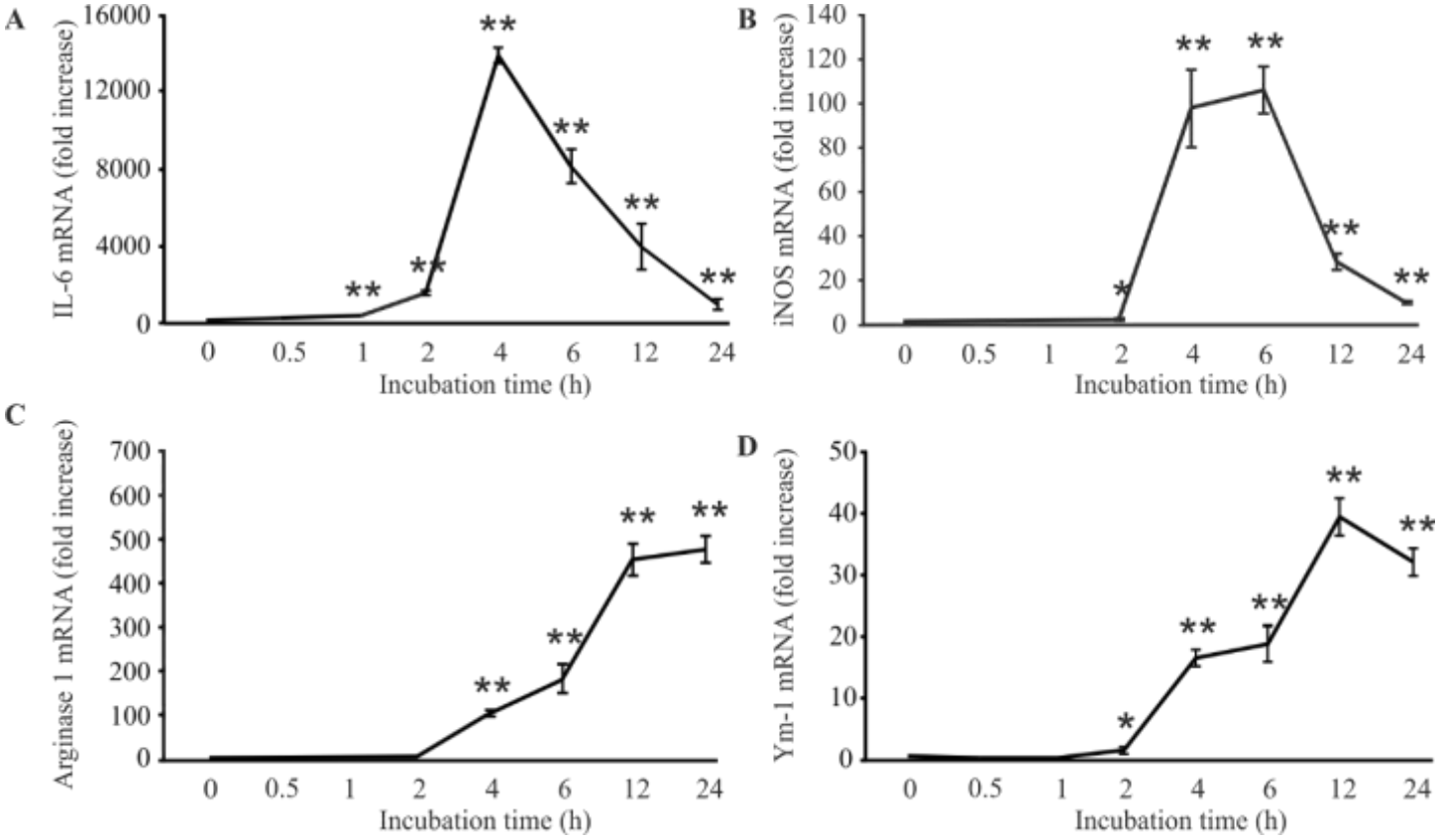

Figure 1: LPS and combination of IL-4 and IL-13 enhanced the expression of markers of the classical M(LPS) and alternative M(IL-4/IL-13) macrophage phenotypes. J774 macrophages were cultured with LPS (10 ng/ml) (A and B) or a combination of IL-4 and IL-13 (both 10 ng/ml) (C-D) for 0.5 – 24 hr to induce the respective phenotypes. mRNA levels of the M(LPS) markers IL-6 (A) and inducible nitric oxide synthase (iNOS) (B), as well as M(IL-4/IL-13) markers arginase 1 (C) and Ym-1 (D) were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. The values were compared to the untreated control (0 h) which was set as 1. The results are expressed as mean ± SEM, n = 4. **: $p < 0.01$ and *: $p < 0.05$, compared to the 0-hr time point.

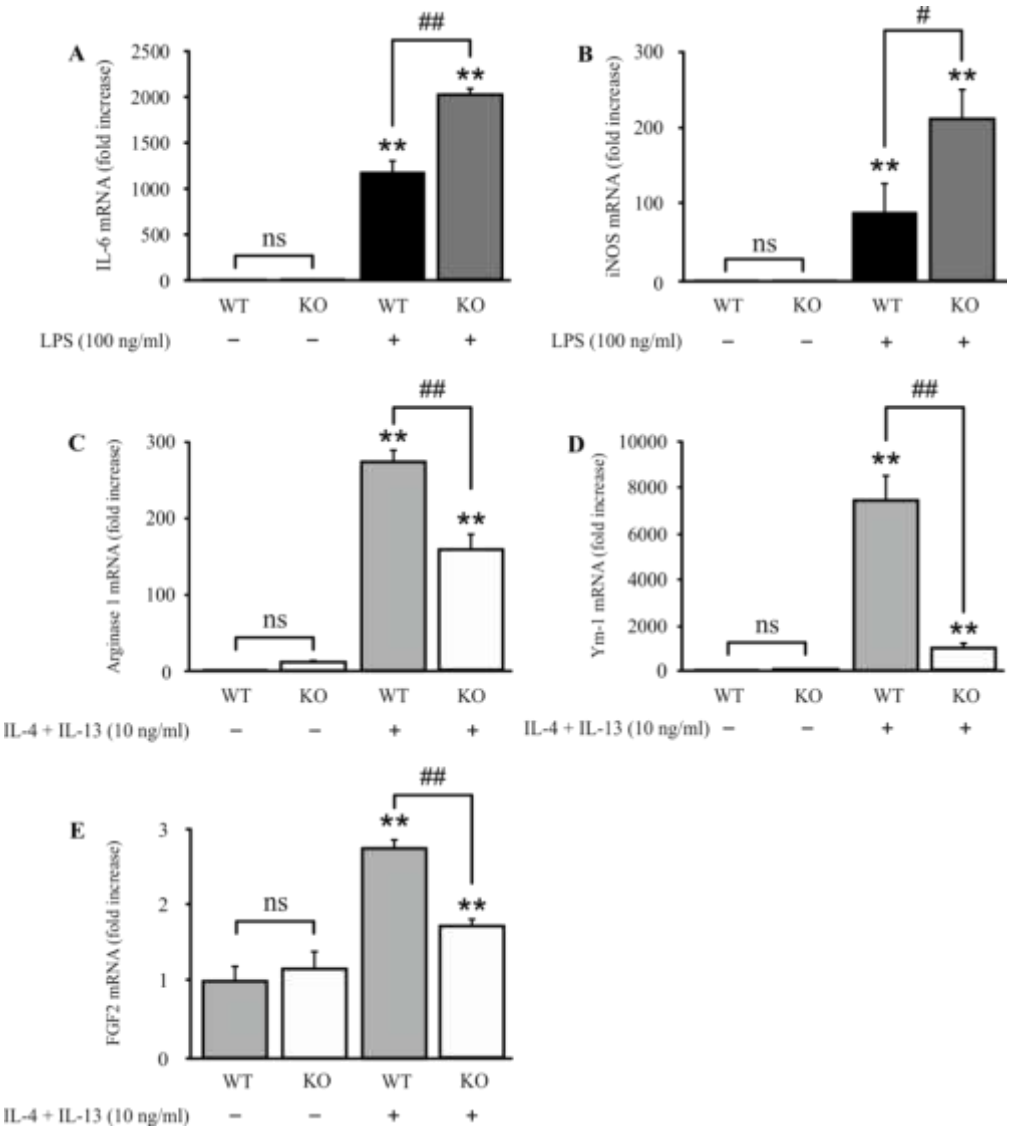

Figure 2. MKP-1 deficiency increased the expression of markers of classical M(LPS) phenotype and decreased the expression of markers of alternative M(IL-4/IL-13) phenotype in murine macrophages. Peritoneal macrophages from wild-type (WT) and MKP-1-deficient (knock-out, KO) mice were cultured with either LPS (100 ng/ml) for 4 hr (A-B), or with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 hr (C-E). mRNA levels of the M(LPS) markers IL-6 (A) and inducible nitric oxide synthase (iNOS) (B), and M(IL-4/IL-13) markers arginase 1 (C), Ym-1 (D) and fibroblast growth factor 2 (FGF2) (E) were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. All values were compared to the WT control, which was set as 1. The results are expressed as mean + SEM, cells from six WT and six KO mice were pooled to

give n = 4. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample. The experiments were repeated three times with similar results.

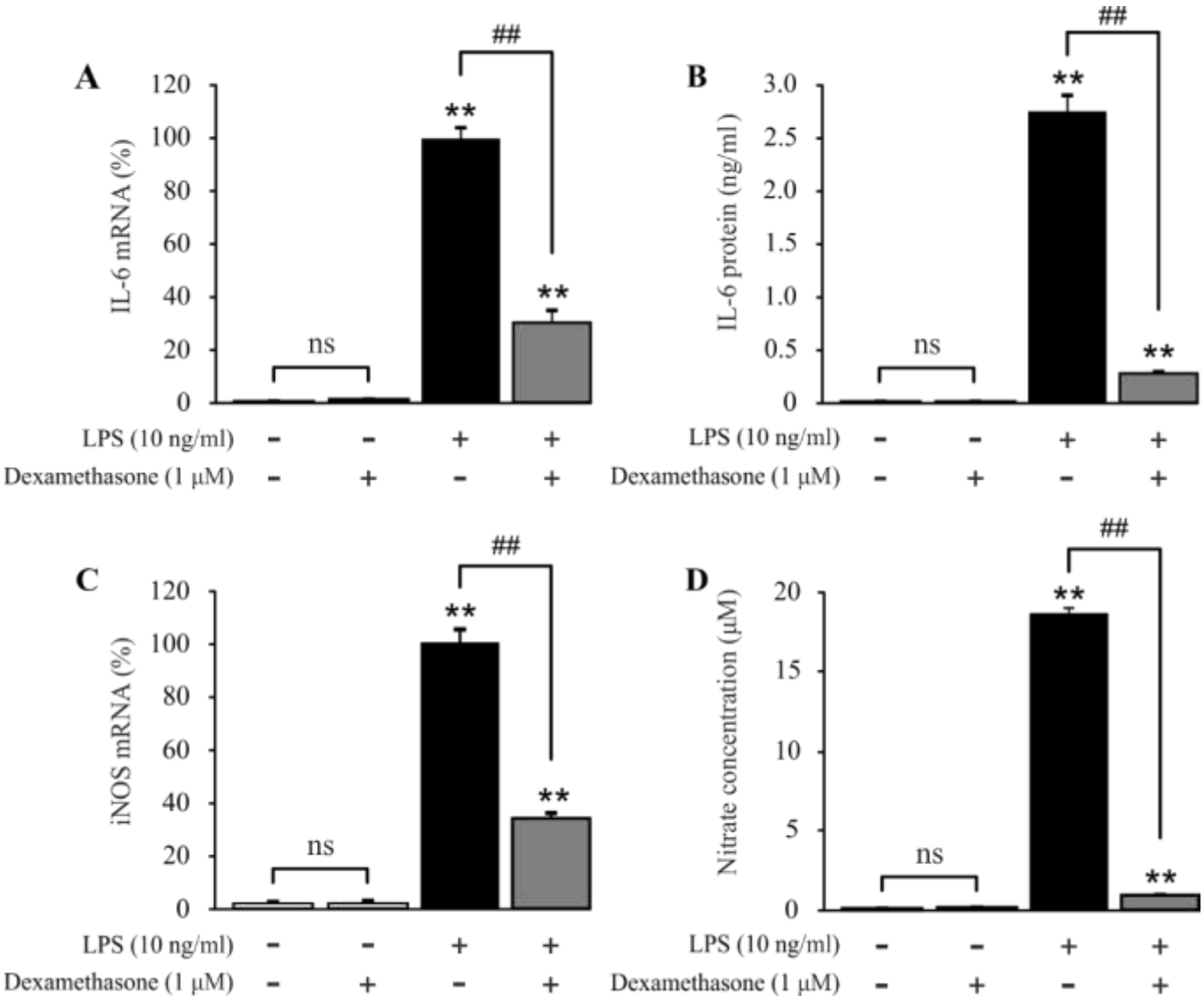

Figure 3: Dexamethasone decreased the expression of markers of pro-inflammatory M(LPS) activation in J774 macrophages. J774 cells were cultured with LPS (10 ng/ml) for 4 hr (A and C) or for 24 hr (B and D), with or without dexamethasone (1 μM). IL-6 (A) and inducible nitric oxide (iNOS) (C) mRNA levels were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. The results were compared against cells treated with LPS alone, which was set as 100 %. IL-6 protein levels were determined

with ELISA (B), and NO production was assessed by measuring the concentration of its stable metabolite nitrite by Griess method (D). The results are expressed as mean + SEM, n = 4. In B, LPS alone raised the IL-6 concentration to 2.8±0.2 ng/ml, and addition of dexamethasone reduced it to 0.3±0.01 ng/ml. In D, LPS alone raised nitrate concentration to 18.6±0.04 μM, and dexamethasone reduced it to 0.9±0.03 μM. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample.

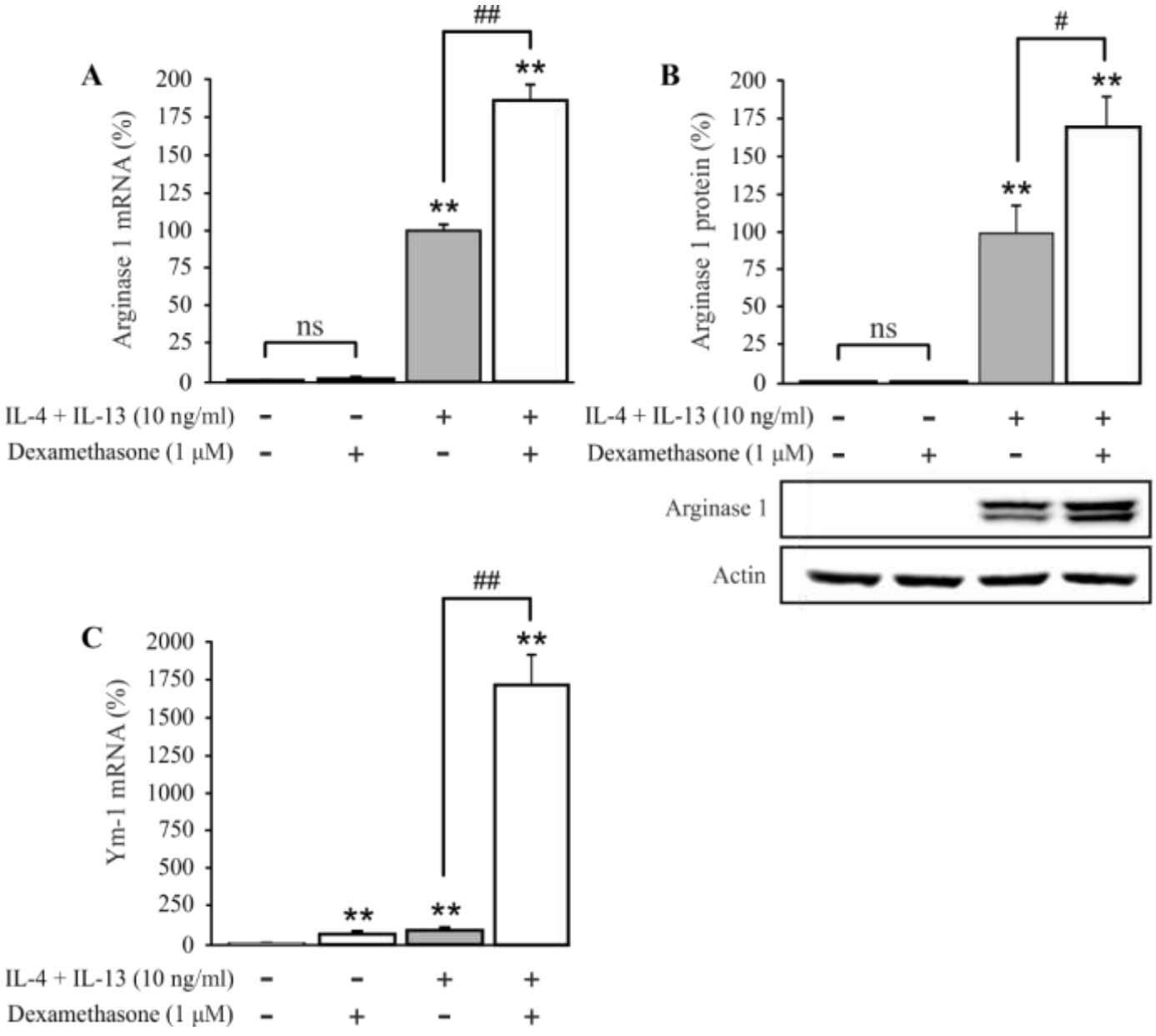

Figure 4: Dexamethasone increased the expression of markers of the anti-inflammatory and healing-promoting M(IL-4/IL-13) activation in J774 macrophages. J774 macrophages were cultured with a combination of IL- 4 and IL-13 (both 10 ng/ml) for 24 hr, with or without dexamethasone (1 µM). Arginase 1 (A) and Ym-1 (C) mRNA levels were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. Arginase 1 protein levels were determined with western blotting (B). All values were compared to the sample treated with IL-4 and IL-13 alone, which was set as 100 %. The results are expressed as mean + SEM, n = 4. **: $p < 0.01$, compared to the untreated control; #: $p < 0.05$, ##: $p < 0.01$ and ns: not significant ($p > 0.05$), compared to the indicated sample. In (B), shown is also a representative western blotting gel of four with similar results.

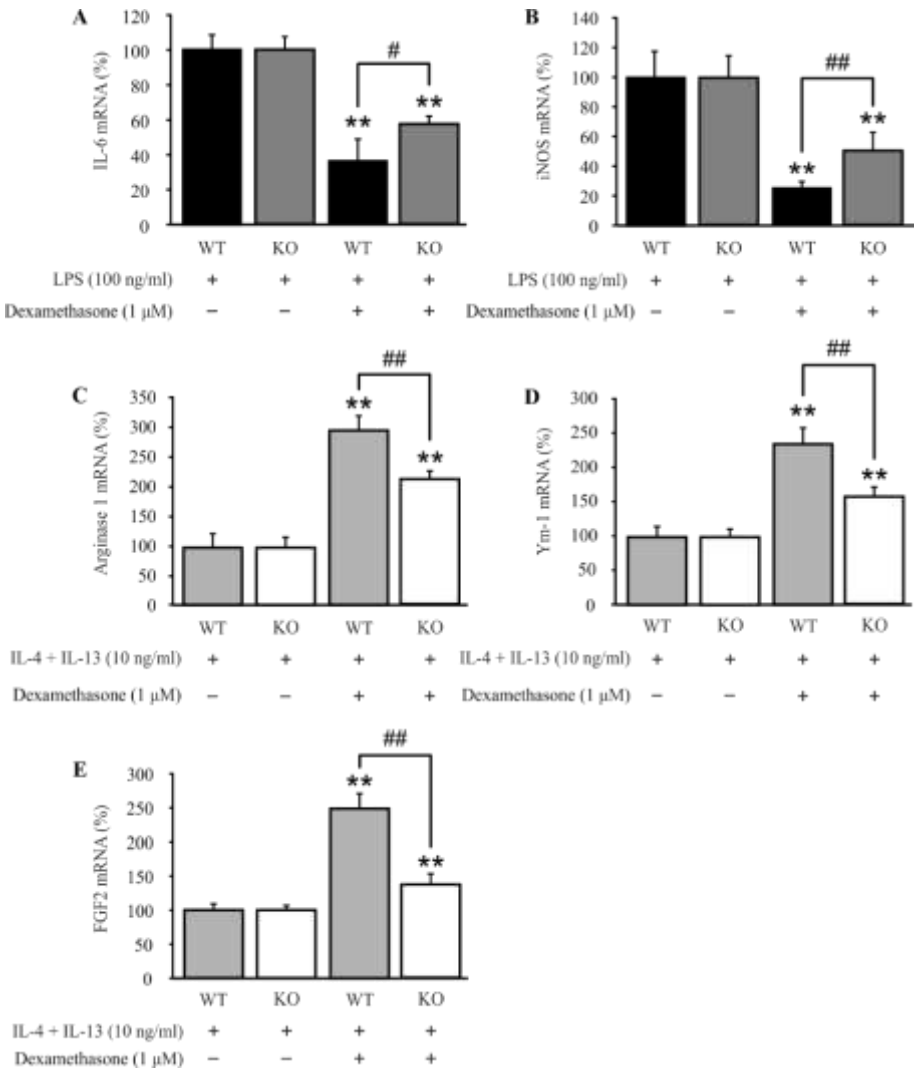

Figure 5. Dexamethasone decreased markers of M(LPS) activation and increased those of M(IL-4/IL-13) activation in macrophages from wild-type mice, and both effects were attenuated in macrophages from MKP-1-deficient mice. Peritoneal macrophages from wild-type (WT) and MKP-1-deficient (knock-out, KO) mice were cultured with either LPS (100 ng/ml) for 4 hr (A and B) or with a combination of IL-4 and IL-13 (both 10 ng/ml) for 24 hr (C-E), in the absence or presence of dexamethasone (1 µM). mRNA levels of the M(LPS) markers IL-6 (A) and inducible nitric oxide synthase (iNOS) (B), and the M(IL-4/IL-13) markers arginase 1 (C), Ym-1 (D) and fibroblast growth factor 2 (FGF2) (E) were measured with quantitative RT-PCR and their expression levels were normalized against GAPDH. WT and KO values were compared separately to the respective samples without dexamethasone, which were set as 100%. The results are expressed as mean + SEM, cells from six WT and six KO mice were pooled to give n = 4. **: $p < 0.01$, compared to the control treated with LPS or IL-4 + IL-13 alone; #: $p < 0.05$ and ##: $p < 0.01$, compared to the indicated sample. The experiments were repeated two times with similar results.

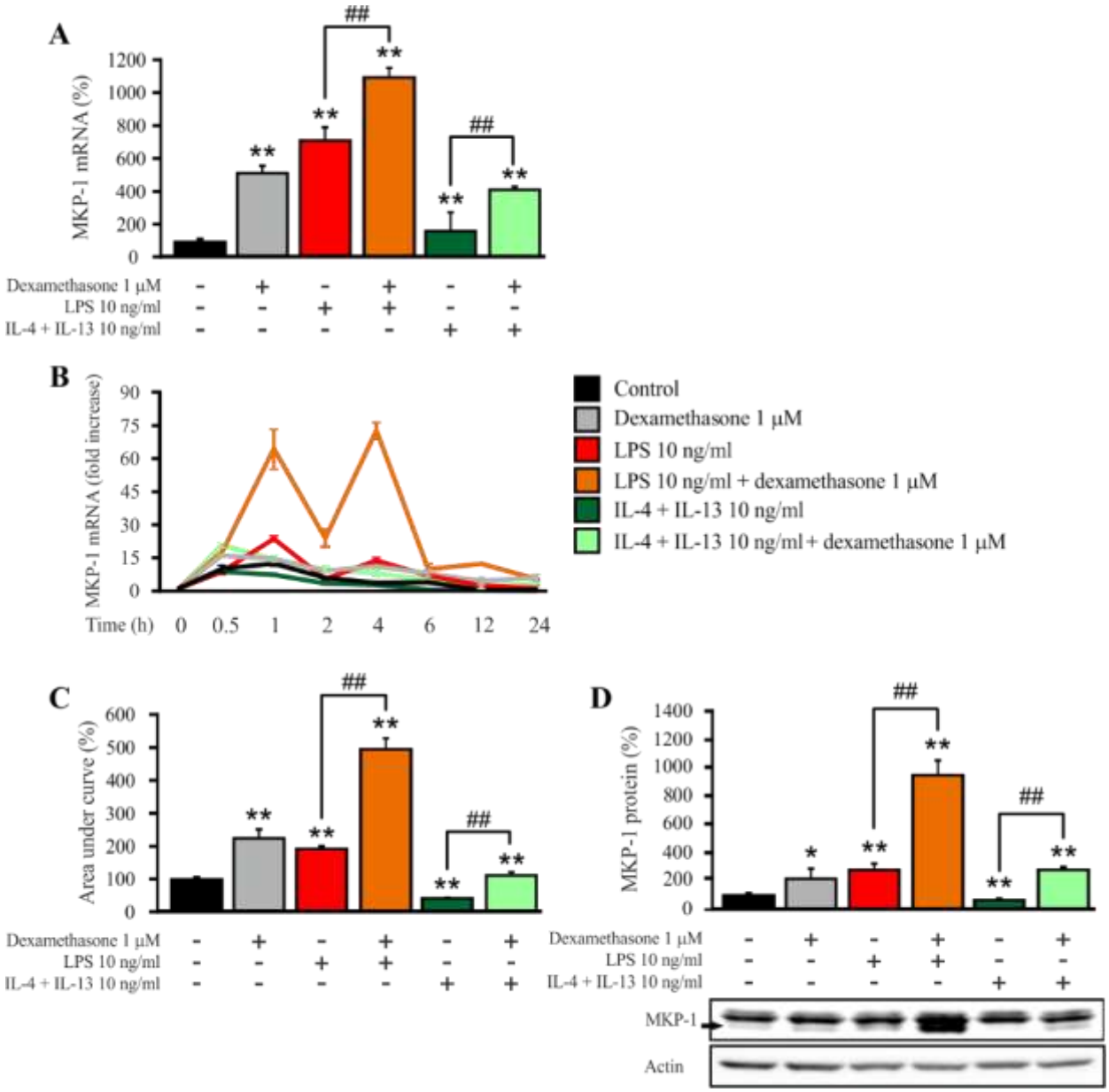

Figure 6. MKP-1 expression was increased by dexamethasone in classically activated M(LPS) and alternatively activated M(IL-4/IL-13) macrophages. (A): Peritoneal macrophages from wild-type mice were incubated for 1 hr with dexamethasone (1 µM), with LPS (10 ng/ml), with IL-4 and IL-13 (10 ng/ml) or with their combinations as indicated, and MKP-1 mRNA was detected by quantitative RT-PCR. (B and C): J774 macrophages were incubated for 0.5 – 24 hr with dexamethasone (1 uM), with LPS (10 ng/ml), with IL-4 and IL-13 (both 10 ng/ml) or with their combinations as indicated. MKP-1 mRNA was detected by quantitative RT-PCR and its expression levels were normalized against GAPDH. (B) shows the time-response curves and (C) indicates total area under the curves of (B), estimated by trapezoidal integration. (D): J774 macrophages were incubated for 1 hr as described in

(A), and MKP-1 protein levels were determined with western blotting and actin was used as a loading control. The results are expressed as mean + SEM, n = 4. **: $p < 0.01$ and*: $p < 0.05$, compared to the control, ##: $p < 0.01$ for indicated comparisons. In (D), shown is also a representative western blotting gel of four with similar results.

Tables

Table 1: Primers and probes used for quantitative RT-PCR

Primer/probe	**Sequence**
mIL-6 forward	5′-TCGGAGGCTTAATTACACATGTTC-3′
mIL-6 reverse	5′-CAAGTGCATCATCGTTGTTCATAC-3′
mIL-6 probe	5′-Fam-CAGAATTGCCATTGCACAACTCTTTTCTCA-TAMRA-3′
miNOS forward	5′-CCTGGTACGGGCATTGCT-3′
miNOS reverse	5′-GCTCATGCGGCCTCCTT-3′
miNOS probe	5′-Fam-CAGCAGCGGCTCCATGACTCCC-TAMRA-3′
mMKP-1 forward	5′-CTCCTGGTTCAACGAGGCTATT-3′
mMKP-1 reverse	5′-TGCCGGCCTGGCAAT-3′
mMKP-1 probe	5′-Fam-CCATCAAGGATGCTGGAGGGAGAGTGTT-TAMRA-3′
mARG1 forward	5′-TCCAAGCCAAAGTCCTTAGAGATTAT-3′
mARG1 reverse	5′-CGTCATACTCTGTTTCTTTAAGTTTTTCC-3′
mARG1 probe	5′-Fam-CGCCTTTCTCAAAAGGACAGCCTCGA-TAMRA-3′
mYm-1 forward	5′-AGTGGGTTGGTTATGACAATGTCA-3′

mYm-1 reverse	5′-GACCACGGCACCTCCTAAATT-3′
mYm-1 probe	5′- Fam-AGCTTCAAGTTGAAGGCTCAGTGGCTCA-TAMRA-3′
mGAPDH forward	5′-GCATGGCCTTCCGTGTTC-3′
mGAPDH reverse	5′-GATGTCATCATACTTGGCAGGTTT-3′
mGAPDH probe	5′-Fam-TCGTGGATCTGACGTGCCGCC-TAMRA-3′

PUBLICATION

II

Attenuating effects of nortrachelogenin on IL-4 and IL-13 induced alternative macrophage activation and on bleomycin-induced dermal fibrosis

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Attenuating Effects of Nortrachelogenin on IL-4 and IL-13 Induced Alternative Macrophage Activation and on Bleomycin-Induced Dermal Fibrosis

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Supporting Information

ABSTRACT: Excessive alternative macrophage activation contributes to fibrosis. We studied the effects of nortrachelogenin, the major lignan component of *Pinus sylvestris* knot extract, on alternative (M2) macrophage activation. J774 murine and THP-1 human macrophages were cultured with IL-4+IL-13 to induce alternative activation, together with the extract and its components. Effects of nortrachelogenin were also studied in bleomycin-induced murine dermal fibrosis model. Knot extract significantly decreased the expression of alternative activation markers—arginase 1 in murine macrophages (97.4 ± 1.3% inhibition at 30 μg/mL) and CCL13 and PDGF in human macrophages—as did nortrachelogenin (94.9 ± 2.4% inhibition of arginase 1 at 10 μM). Nortrachelogenin also decreased PPARγ expression but had no effect on STAT6 phosphorylation. In vivo, nortrachelogenin reduced bleomycin-induced increase in skin thickness as well as the expression of collagens COL1A1, COL1A2, and COL3A1 (all by >50%). In conclusion, nortrachelogenin suppressed IL-4+IL-13-induced alternative macrophage activation and ameliorated bleomycin-induced fibrosis, indicating therapeutic potential in fibrosing conditions.

KEYWORDS: *alternative macrophage activation, nortrachelogenin, Pinus sylvestris, fibrosis, bleomycin*

INTRODUCTION

Macrophages are important and versatile immune effector cells, performing a number of homeostatic and inflammation-related functions. These cells fight infections by phagocytizing pathogens and presenting antigens to T cells and thus act as mediators between innate and adaptive immunity. They also regulate inflammation by secreting cytokines and other soluble factors. These factors can either promote or inhibit inflammation, depending on the environment and the stage of inflammatory response. Today it is widely accepted that the macrophages performing subsets of these tasks actually display different phenotypes, generally grouped into the "classical" (M1) and "alternative" (M2) activation modes.[1]

Classical macrophage activation is induced during inflammation and immune response and further promotes these processes, while alternatively activated cells contribute to tissue healing and resolution of inflammation.[2] On the other hand, activated macrophages play a role in the development of many inflammatory diseases. For instance, classically activated macrophages have been implicated in autoimmunity,[3] while alternatively activated cells are key players in fibrotic diseases such as pulmonary fibrosis,[4] chronic pancreatitis,[5] and systemic sclerosis (scleroderma).[6,7] While various anti-inflammatory agents attenuate classical macrophage activation,[8] current treatment options for fibrotic diseases are severely limited, with no disease-modifying agents available for states such as systemic sclerosis.[9] There is thus a major unmet need for medications capable of effectively affecting the development of fibrosis.

A prominent type of alternative macrophage activation is induced by major type 2 T helper cell cytokines interleukin 4 (IL-4) and interleukin 13 (IL-13). This phenotype is mediated by signal transducer and activator of transcription 6 (STAT6)[1] and peroxisome proliferator activated receptors (PPARs).[10,11] Arginase 1 is a canonical marker of this type of alternative activation in murine macrophages. While classically activated macrophages express inducible nitric oxide synthase (iNOS) to produce the cytotoxic and pro-inflammatory mediator nitric oxide (NO) from arginine,[12] alternatively activated macrophages use arginase to convert arginine into L-proline and polyamines, precursors for extracellular matrix synthesis.[1] Human markers of IL-4+IL-13-induced alternative activation include the chemokine (C−C motif) ligand 13 (CCL13) and platelet-derived growth factor beta (PDGFB). CCL13 is an immunoregulatory chemokine implicated in Th2/M2 functions such as allergic inflammation.[13,14] PDGFB exists as a dimeric glycoprotein, either as a homodimer (PDGF-BB) or as a heterodimer with PDBFA (PDGF-AB). It potently stimulates the proliferation of fibroblasts and other cells important for tissue growth and healing and has been implicated as a causal factor in fibrotic disease.[15,16]

Lignans are bioactive polyphenolic compounds synthesized in plants as secondary metabolites. Plant extracts rich in these compounds have traditionally been used in folk medicine,[17] and

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they have attracted considerable interest due to their potential beneficial effects on various inflammatory conditions.[18] Extracts from tree knots (the parts of branches embedded in the stem) have previously been shown to be rich sources of lignans.[19,20] Our previous studies with knot extract from Scots pine (*Pinus sylvestris*) and its major active components, stilbenes of the pinosylvin type and the predominant lignan component, nortrachelogenin (Figure 1), have confirmed this extract to be

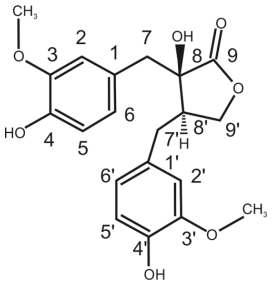

Figure 1. Chemical structure of nortrachelogenin.[30]

a rich source of anti-inflammatory and immunomodulatory compounds.[21,22] Antibacterial and antifungal properties have also been reported.[23,24] *Pinus sylvestris* products have traditionally been used as natural anti-inflammatory agents in the treatment of arthritis and related conditions, as well as a topical remedy for various skin and scalp disorders.[25,26] The anti-inflammatory lignans have previously been found to protect animals in models of experimental cerebral ischemia-reperfusion injury,[27] carrageenan-induced paw inflammation,[22] and sepsis.[28] Interestingly, they have also been found to inhibit Th2 cell-mediated allergic lung inflammation.[29] However, as far as we are aware, the potential effects of nortrachelogenin and other lignans on alternative macrophage activation have remained largely unexplored.[30]

In the present study, we showed that *Pinus sylvestris* knot extract and its major but underinvestigated bioactive component nortrachelogenin reduce IL-4+IL-13-induced alternative macrophage activation. The results were also translated to in vivo, as nortrachelogenin was found to reduce bleomycin-induced dermal fibrosis in mice.

■ MATERIALS AND METHODS

Chemicals. Purified lignans conidendrin (purity 95%), matairesinol (purity 95%), nortrachelogenin (purity 87%), pinoresinol (purity 95%), and secoisolariciresinol (purity 97%) were purchased from Arbonova (Turku, Finland), and hydroxymatairesinol (purity >90%) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pinosylvin (purity 99%) and monomethyl pinosylvin (purity 99%) were from Sequoia Research Products (Pangbourne, U.K.), abietic acid (purity >90%) was from Alfa Aesar (Haverhill, MA, U.S.A.), and pimaric acid was from MP Biomedicals (Santa Ana, CA, U.S.A.).

Rabbit polyclonal arginase 1 (sc-18351), STAT6 (sc-981), and β-actin (sc-1615R) antibodies, along with goat HRP-conjugated antirabbit polyclonal antibody (sc-2004) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). pSTAT6 antibody (ab54461) was purchased from Abcam (Cambridge, U.K.). Other reagents, unless otherwise indicated, were from Sigma Chemical Co.

***Pinus sylvestris* Knotwood Extract.** A knotwood extract of Scots pine (*Pinus sylvestris*) was prepared and analyzed as described previously.[20] In brief, wood material was obtained from a healthy Scots pine growing in the Southern part of Finland. Ground dry knot material was extracted in an accelerated solvent extractor (ASE) apparatus in two stages: with hexane at 90 °C (3 × 5 min) to remove most of the lipophilic extractives and with ethanol/water (95:5 by vol) at 100 °C (3 × 5 min). The second extract, which contained mainly the hydrophilic extractives, was evaporated in a vacuum to dryness and used in the experiments. Analysis of the extract was carried out by gas chromatography−mass spectrometry after silylation (Figure S1). In cell culture experiments, the extract was added into the culture medium at the final concentration of 100 μg/mL, yielding 19 μM nortrachelogenin, 5.6 μM matairesinol, 24 μM pinosylvin, 56 μM monomethyl pinosylvin, 13 μM abietic acid, 13 μM dehydroabietic acid, and 10 μM pimaric acid.

Cell Culture. Murine J774 macrophages (obtained from ATCC, Manassas, VA, U.S.A.) were cultured at 37 °C in humidified air and in CO_2 (5%) atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%) (Lonza Verviers SPRL, Verviers, Belgium), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (all three from Invitrogen, Paisley, U.K.). Cells were harvested using trypsin/EDTA and seeded on 24-well plates. Confluent cultures were treated with fresh culture medium containing the compounds of interest. Human THP-1 cell line (ATCC) was cultured according to a similar protocol, except that the medium used was RPMI 1640 containing L-glutamine (2 mM), sodium pyruvate (1 mM), 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES) (10 mM), glucose (4.5 g/L), and sodium bicarbonate (1.5 g/L) and supplemented with 10% fetal bovine serum (all from Lonza); penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (all from Invitrogen); and 2-mercaptoethanol (50 μM). Cells were then seeded on 24-well plates and differentiated into macrophages by adding PMA (phorbol myristate acetate, 100 nM) 72 h before starting the experiments. Confluent cultures were then exposed to fresh culture medium containing the compounds of interest. The alternative macrophage activation was induced with interleukins 4 and 13, both 10 ng/mL, and the incubations were continued for 24 h.

Cell Viability. Modified XTT method (Cell Proliferation kit II, Roche Diagnostics, Indianapolis, IN, U.S.A.) was used to investigate cell viability. Pine knot extract and nortrachelogenin, in the concentrations and conditions used in this study, were not found to evoke cytotoxicity in either J774 or THP-1 cells (Figure S2). Also, no histological signs of tissue necrosis were seen in nortrachelogenin-treated skin (see Figure 7A).

Sample Preparation and Western Blotting. Cell lysates for arginase 1 Western blotting and nuclear extracts for pSTAT6 Western blotting were prepared as previously described,[31] and protein contents of the samples were measured using the Coomassie blue method.[32] Western blotting was carried out as previously described.[33]

ELISA. After 24 h, incubations were terminated by collecting the cell culture media. Culture medium samples were stored at −20 °C until analyzed. The concentrations of human CCL13, murine IL-6, and murine TNFα were determined by enzyme-linked immunosorbent assay (ELISA) by using reagents from R&D Systems Europe Ltd., Abingdon, U.K. (Catalog nos. DY327, DY406, and DY410, respectively). The detection limits were 3.8 pg/mL (hCCL-13), 7.8 pg/mL (mIL-6), and 15.6 pg/mL (mTNF).

Nitrite Measurement with Griess Method. Nitric oxide (NO) production was evaluated by determining the concentration of nitrite, a stable product of NO in aqueous environment, in the culture medium by the Griess reaction.[34]

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted with the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) according to manufacturer's instructions. Reverse transcription to cDNA was carried out using TaqMan Reverse Transcription kit (cell line samples) and Maxima First Strand cDNA Synthesis kit (murine skin samples) (both from Applied Biosystems, Foster City, CA, U.S.A.) with 500 μg of DNA per sample. Primers and probes for mouse arginase 1 (NCBI Reference Sequence NM007482.3) and iNOS (BC062378.1) as well as human (NM002046.6) and murine (BC083065.1) glyceraldehyde 3-phosphate dehydrogenase (GAPDH, used as a control gene) were designed using Primer Express Software

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Table 1. Primers and Probes Used for Quantitative RT-PCR

primer/probe	concentration	sequence
mGAPDH forward	300 nM	5′-GCATGGCCTTCCGTGTTC-3′
mGAPDH reverse	300 nM	5′-GATGTCATCATACTTGGCAGGTTT-3′
mGAPDH probe	150 nM	5′-Fam-TCGTGGATCTGACGTGCCGCC-TAMRA-3′
mARG1 forward	900 nM	5′-TCCAAGCCAAAGTCCTTAGAGATTAT-3′
mARG1 reverse	300 nM	5′-CGTCATACTCTGTTTCTTTAAGTTTTTCC-3′
mARG1 probe	200 nM	5′-Fam-CGCCTTTCTCAAAAGGACAGCCTCGA-TAMRA-3′
miNOS forward	300 nM	5′-CCTGGTACGGGCATTGCT-3′
miNOS reverse	300 nM	5′-GCTCATGCGGCCTCCTT-3′
miNOS probe	150 nM	5′-Fam-CAGCAGCGGCTCCATGACTCCC-TAMRA-3′
hGAPDH forward	300 nM	5′-AAGGTCGGAGTCAACGGATTT-3′
hGAPDH reverse	300 nM	5′-GCAACAATATCCACTTTACCAGAGTTAA-3′
hGAPDH probe	150 nM	5′-CGCCTGGTCACCAGGGCTGC-3′

(Applied Biosystems, Foster City, CA, U.S.A.) and supplied by Metabion (Martinsried, Germany) (Table 1). TaqMan Gene Expression assays for human CCL13 (Hs01033504_g1) and PDGFB (Hs00966522_m1) as well as mouse PPARγ (Mm01184322_m1), FN1 (Mm01256744_m1), IL-13 (Mm00434204_m1), COL1A1 (Mm00801666_g1), COL1A2 (Mm00483888_m1), and COL3A1 (Mm01254476_m1) were from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[33] Average GAPDH CT values (to confirm the stability of reference gene expression upon treatment with pine knot extract and nortrachelogenin) are provided in Figure S3.

Mouse Model of Bleomycin-Induced Dermal Fibrosis. Male C57BL/6N mice (Scanbur Research A/S, Karlslunde, Denmark), aged 10 weeks, were used to study the effects of nortrachelogenin in bleomycin-induced dermal fibrosis. Mice were housed in the animal facility of the University of Tampere under standard conditions (12/12 light/dark cycle, +22 ± 1 °C temperature, 50−60% humidity) with water and food ad libitum. The study was approved by the National Animal Experiment Board, and the experiments were carried out in accordance with the EU legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU).

The upper dorsa of mice were shaved, and a square (∼1.5 cm^2) was drawn with a marker. Treatment groups consisting of eight mice each were anesthetized with sevoflurane inhalation and injected with 500 μg of bleomycin (Cayman Chemical, Michigan, U.S.A.) in 100 μL of PBS, with or without 250 μg of nortrachelogenin every other day for 21 days. Uninjected male C57BL/6N mice from the same lineage were used as controls. The injection site in the defined shaved area was rotated, i.e., first four injections were administered into the corners of the square with the fifth injection being delivered into the middle of the square. On the day after the last injection, the mice were euthanized with CO_2, followed by immediate cervical dislocation. The injected skin (marked square created on the dorsal surface) was cut with a punch of 6 mm of diameter. Skin specimens were fixed in formalin (10%, 24 h) and processed for histological analyses or stored in RNA*later* solution (Invitrogen, Life Technologies, Carlsbad, CA, U.S.A.) and processed for RNA extraction.

In the histological analysis, 6 μm sections of skin were cut, mounted on a slide, and stained with hematoxylin and eosin (HE) (Histolab Products AB, Göteborg, Sweden). Dermal thickness was measured in HE-stained sections by measuring the distance between the epidermal−dermal junction and the dermal-fat junction at 6 different locations in each section using the ImageJ program (National Institutes of Health, Bethesda, MD, U.S.A.).

Statistics. Results are expressed as mean ± standard error of mean (SEM). One-way analysis of variance was used with Dunnett's or Bonferroni's multiple testing correction where appropriate. Differences were considered significant at $p < 0.05$. Data were analyzed using the Prism computerized package (Graph Pad Software, San Diego, CA, U.S.A.).

RESULTS

Effects of Pine Knot Extract and Nortrachelogenin on IL-4+IL-13-Induced Arginase 1 Expression in Murine J774 Macrophages. First, we examined the effects of the pine knot extract and its main constituents on the expression of arginase 1, a canonical marker of IL-4+IL-13-induced alternative macrophage activation. Unstimulated J774 cells expressed arginase 1 at relatively low levels, but stimulation with IL-4 and IL-13 significantly upregulated this factor. In these alternatively activated J774 macrophages, the pine knot extract significantly reduced arginase 1 mRNA levels as compared to the control. Nortrachelogenin, the major lignan constituent of the extract, had a similar effect. This effect was shared by matairesinol but not by the other major constituents (Figure 2A).

Macrophages can change their phenotype according to environmental signals, and induction of classical (M1) macrophage activation by nortrachelogenin could therefore explain the observed effects. To rule this out, we also studied the effects of the knot extract and nortrachelogenin on production of IL-6, nitrite, and TNFα, three established markers of classical M1 activation,[35] in the conditions described above. Neither of these M1 markers were increased by the knot extract or nortrachelogenin (Figure S4).

To study whether the effect of nortrachelogenin was commonly shared by the lignan family, we investigated the effects of a series of lignans known to be present in plant extracts. Nortrachelogenin and matairesinol significantly reduced arginase 1 expression in alternatively activated macrophages. In contrast, hydroxymatairesinol, pinoresinol, secoisolariciresinol, and conidendrin, when used at 30 μM concentrations, did not significantly affect arginase 1 expression (Figure 2B).

The data above suggest that nortrachelogenin significantly contributes to the discovered bioactivity of the pine knot extract to reduce alternative macrophage activation. Next, we studied dose-dependence of the effects of nortrachelogenin on arginase 1 expression. As shown in Figure 3, both knot extract and nortrachelogenin decreased arginase 1 expression in a dose-dependent manner.

Effects of Pine Knot Extract and Nortrachelogenin on IL-4+IL-13-Induced Expression of CCL13 an PDGFB in Human THP-1 Macrophages. To test whether pine knot extract and nortrachelogenin could suppress IL-4+IL-13-induced alternative activation also in human macrophages, we studied their effects on the expression of CCL13 and PDGFB, which have been recognized as markers of alternative activation in human macrophages.[16,36] Resting THP-1 macrophages

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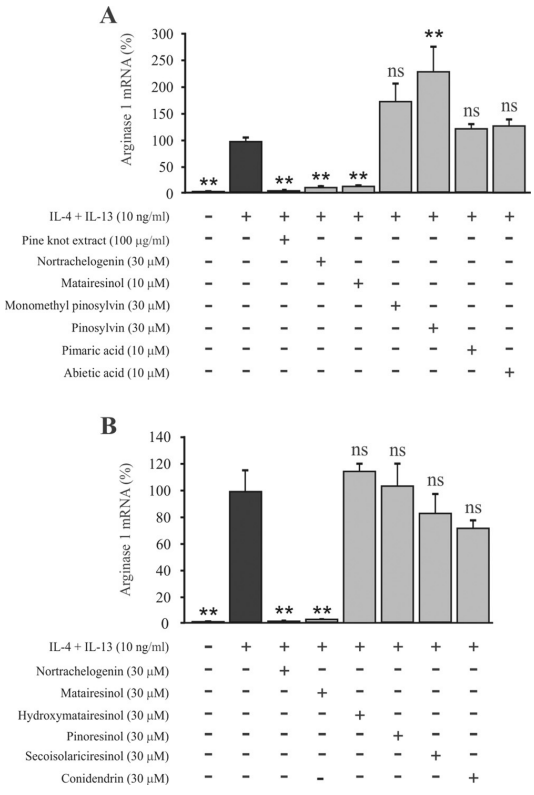

Figure 2. Effects of pine knot extract and its major components (A), as well as a series of naturally occurring lignans (B) on arginase 1 expression in IL-4 and IL-13-treated alternatively activated macrophages. J774 murine macrophages were cultured for 24 h with IL-4+IL-13, together with the pine knot extract or its main constituents (used at concentrations close to those present in the extract at 100 μg/mL) (A) or indicated lignans (B). Arginase 1 mRNA levels were determined with quantitative RT-PCR and normalized to GAPDH. Results are shown as mean ± SEM, with cells cultured with IL-4 and IL-13 alone set to 100%. $n = 3$. $^{**}p < 0.01$, compared with cells cultured with IL-4 and IL-13 alone. ns = not significant.

expressed CCL13 and PDGFB at relatively low levels. Incubating the cells with IL-4 and IL-13 for 24 h to induce alternative activation significantly enhanced the expression of PDGFB and CCL13. In IL-4+IL-13-stimulated THP-1 macrophages, pine knot extract and nortrachelogenin reduced CCL13 expression in a dose-dependent manner (Figure 4). They also reduced IL-4+IL-13-induced PDGFB expression. (Figure 5).

Effects of Pine Knot Extract and Nortrachelogenin on IL-4+IL-13-Induced PPARγ Expression and STAT6 Phosphorylation in Murine J774 Macrophages. To evaluate the mechanisms by which pine knot extract and nortrachelogenin may regulate IL-4+IL-13-induced alternative activation of macrophages, we studied their effects on STAT6 phosphorylation[1] and PPARγ expression.[11] Pine knot extract or nortrachelogenin exerted no significant effect on STAT6 phosphorylation as measured by Western blotting (Figure 6A). However, both of them decreased the expression of PPARγ in a statistically significant manner (Figure 6B), suggesting that the effects of pine knot extract and nortrachelogenin are, at least partly, mediated through downregulation of PPARγ.

Effects of Nortrachelogenin in Bleomycin-Induced Skin Fibrosis. Alternative macrophage activation has been shown to contribute to the development of fibrotic dermal conditions, including a bleomycin-induced model of scleroderma.[37] Therefore, to study the effects of nortrachelogenin in vivo, we decided to evaluate its effects in this model. First, we quantified skin fibrosis by determining the dermal thickness using histological analysis. Nortrachelogenin partially reversed bleomycin-induced increase of skin thickness (Figure 7A and B).

Increased collagen production and deposition is a central feature of bleomycin-induced dermal fibrosis.[38] Thus, we evaluated the gene expression of collagens in skin after administration of bleomycin and nortrachelogenin. As illustrated in Figure 7C−E, the mRNA expressions of collagens COL1A1, COL1A2, and COL3A1 were enhanced in bleomycin-induced fibrosis. Treatment with nortrachelogenin significantly reduced these three collagen subtypes.

Also, skin expression of arginase 1, a marker of M2 activation, was increased by bleomycin. The effect was partially reversed by nortrachelogenin (Figure 7F), suggesting that reduced alternative macrophage activation contributes to the beneficial effect of nortrachelogenin in bleomycin-induced model of skin fibrosis. On the other hand, nortrachelogenin treatment did not reverse bleomycin-induced changes in the expression of inflammatory factors iNOS and IL-13 or fibronectin (Figure S5).

■ DISCUSSION

In the present study, we investigated the effects of *Pinus sylvestris* knot extract and its major lignan component nortrachelogenin on IL-4+IL-13-induced alternative macrophage activation. *Pinus sylvestris* knot extract attenuated alternative activation in both human and murine macrophages as measured by decreased expression of markers of this phenotype. Similar effects were observed for nortrachelogenin, suggesting that this compound is at least in part responsible for the observed biological effects of the pine knot extract. The effects of nortrachelogenin were also studied in bleomycin-induced dermal fibrosis in mice, a disease model known to be driven by alternatively activated (M2) macrophages. In this model, nortrachelogenin decreased collagen COL1A1, COL1A2, and COL3A1 expression, resulting in less severe skin fibrosis.

In their "native" role, lignans function as a part of a plant's immune system, possessing antibacterial and antifungal properties.[23] In recent years, some attention has been paid to their potentially beneficial effects in mammals. Lignans have been found to possess anti-inflammatory effects that are mediated through inhibition of NF-κB signaling and COX-2 expression,[27] as well as to display protective effects in animal models of acute carrageenan-induced paw edema,[22] cerebral ischemia-reperfusion injury,[27] and sepsis.[28] They have also been shown to inhibit Th2 cell-mediated allergic lung inflammation.[29]

Lignans in general,[27] and nortrachelogenin in particular,[22] have previously been shown to regulate classical macrophage activation. These cells coordinate the inflammatory response and drive the pathogenesis of various inflammatory diseases.[1] Therefore, the aforementioned beneficial effects of lignans, at least partly, can reasonably be attributed to downregulation of classical macrophage activation. However, as far as we are aware, almost nothing is known about their effects on alternative macrophage activation. This is the first study to demonstrate

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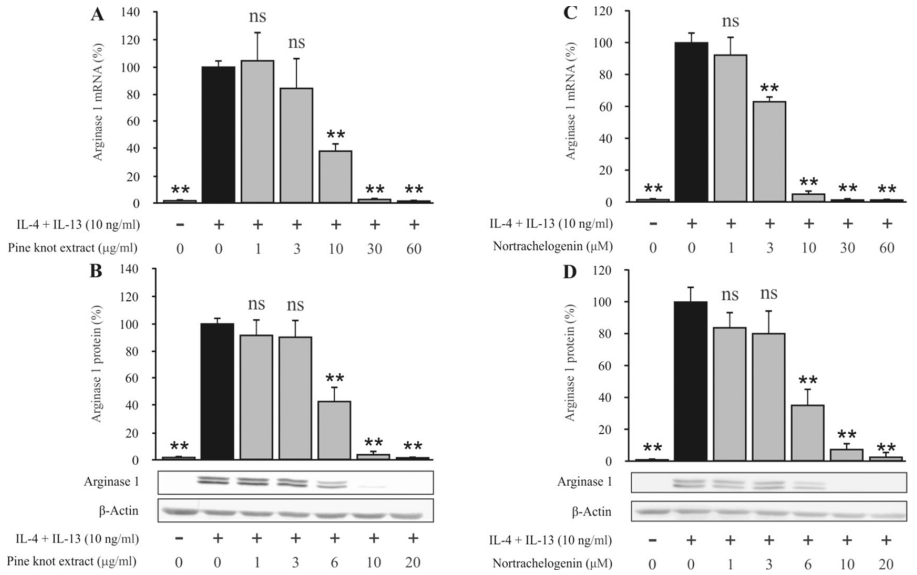

Figure 3. Dose-dependent effect of pine knot extract (A, B) and nortrachelogenin (C, D) on arginase 1 expression in IL-4 and IL-13-treated alternatively activated macrophages. Murine J774 macrophages were incubated for 24 h with IL-4 and IL-13, together with increasing concentrations of the pine knot extract (A, B) or nortrachelogenin (C, D). Arginase 1 mRNA levels were determined with quantitative RT-PCR and normalized to GAPDH (A, C) and protein levels with Western blot, using β-actin as a loading control (B, D). Results are shown as mean ± SEM, with cells cultured with IL-4 and IL-13 alone set to 100%. $n = 4$. $^{**}p < 0.01$, compared with cells cultured with IL-4 and IL-13 alone. ns = not significant.

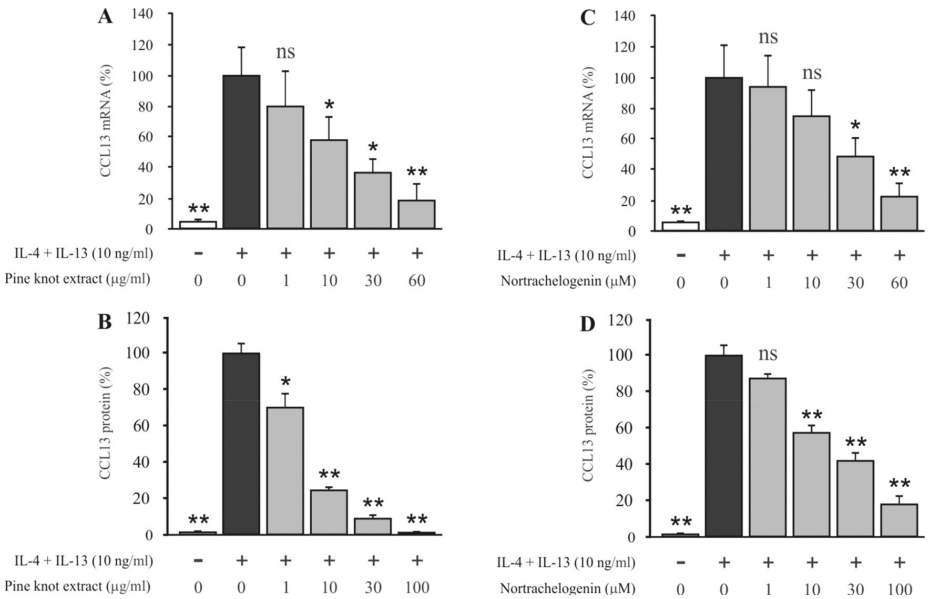

Figure 4. Dose-dependent effect of pine knot extract (A, B) and nortrachelogenin (C, D) on CCL13 expression in IL-4 and IL-13-treated alternatively activated human macrophages. THP-1 cells were incubated for 24 h with IL-4 and IL-13, together with increasing concentrations of the pine knot extract (A, B) or nortrachelogenin (C, D). CCL13 mRNA levels, normalized to GAPDH, were determined with quantitative RT-PCR (A, C) and protein concentrations in the culture media with ELISA (B, D). Results are shown as mean ± SEM, with cells cultured with IL-4 and IL-13 alone set to 100%. $n = 4$. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with cells cultured with IL-4 and IL-13 alone. ns = not significant.

that a lignan compound can significantly suppress IL-4+IL-13-induced alternative macrophage activation in vitro and retard the development of experimentally induced skin fibrosis in vivo. Nortrachelogenin was found to downregulate fibrosis-related

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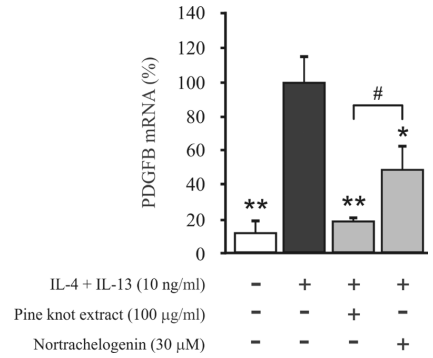

Figure 5. Effects of pine knot extract and nortrachelogenin on PDGFB expression in IL-4 and IL-13-treated alternatively activated human macrophages. THP-1 cells were cultured for 24 h in the presence of IL-4 and IL-13, with or without the pine knot extract or nortrachelogenin. PDGFB mRNA levels, normalized to GAPDH, were determined with quantitative RT-PCR. Results are shown as mean ± SEM, with cells cultured with IL-4 and IL-13 alone set to 100%. $n = 4$. *$p < 0.05$ and **$p < 0.01$, compared with cells cultured with IL-4 and IL-13 alone. #$p < 0.05$ for the indicated comparison.

markers of alternative macrophage activation, but more research is needed to further elucidate the mechanism of action of nortrachelogenin in alternatively activated macrophages and other cell types involved in fibrosis, including fibroblasts and myofibroblasts.

In M1 macrophages, nortrachelogenin has previously been shown to inhibit IL-6/STAT3 signaling[39] as well as the PI3K/Akt signaling pathway, promoting apoptosis of tumor cells.[40] In our data, whole pine knot extract and nortrachelogenin decreased PPARγ expression in IL-4+IL-13-stimulated macrophages, suggesting that their effects on alternative macrophage activation are at least partly mediated through this regulatory factor.[11] Interestingly, knot extract and nortrachelogenin did not affect phosphorylation of STAT6, often considered the seminal regulator of alternative macrophage activation. However, STAT6 and PPARγ seem to drive alternative macrophage activation in a synergistic but independent manner. For instance, Odegaard and co-workers have demonstrated that IL-4-induced STAT6 phosphorylation remains unaltered in PPARγ knockout animals while alternative M2 macrophage activation is significantly reduced, which supports the present data.[10]

IL-4+IL-13-induced alternatively activated macrophages play a role in tissue healing. They produce extracellular matrix precursors from arginine via ornithine[41] and also direct the actions of other cell types such as fibroblasts and myofibroblasts by producing profibrotic mediators.[42] On the other hand, when excessive and/or dysregulated, these processes might reasonably be expected to lead to fibrosis. The literature lends support to this hypothesis: alternatively activated macrophages have been shown to be present in large numbers in various fibrotic states such as dermal sclerosis, chronic pancreatitis, and idiopathic pulmonary fibrosis.[4−6] This is also in line with the present finding that arginase 1 expression is enhanced in bleomycin-induced skin fibrosis. Disease-modifying treatments for fibrotic diseases are currently severely limited,[9] and modulating alternative macrophage activation could be a promising approach for meeting this unmet therapeutic need.

Bleomycin-induced dermal fibrosis has been shown to mimic many of the essential features of fibrotic skin disease, such as increased production of extracellular matrix components.[43] In our data, nortrachelogenin reduced dermal thickness in bleomycin-induced dermal fibrosis, also downregulating the expression of several collagens and the alternative M2 macrophage activation marker arginase 1. This suggests that nortrachelogenin might have a beneficial effect against fibrosing conditions. In addition to its beneficial effect in fibrosing processes, suppression of alternative macrophage activation may

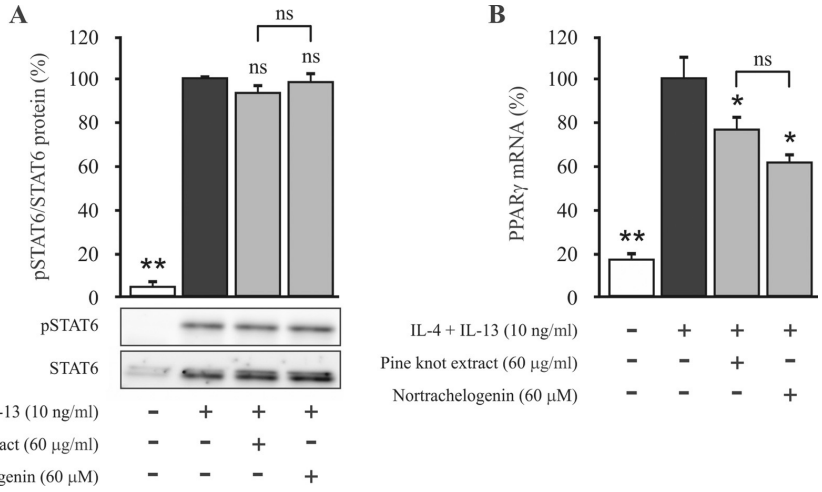

Figure 6. Effects of pine knot extract and nortrachelogenin on STAT6 phosphorylation (A) and PPARγ expression (B) in IL-4 and IL-13-treated alternatively activated macrophages. Murine J774 macrophages were cultured for 30 min (A) or 2 h (B) in the presence of IL-4 and IL-13, with or without pine knot extract or nortrachelogenin. pSTAT6 protein levels, normalized to total STAT6, were determined with Western blot (A) and PPARγ mRNA levels, normalized to GAPDH, with RT-PCR (B). Results are shown as mean ± SEM, with cells cultured with IL-4 and IL-13 alone set to 100%. $n = 4$. *$p < 0.05$ and **$p < 0.01$, compared with cells cultured IL-4 and IL-13 alone. ns = not significant.

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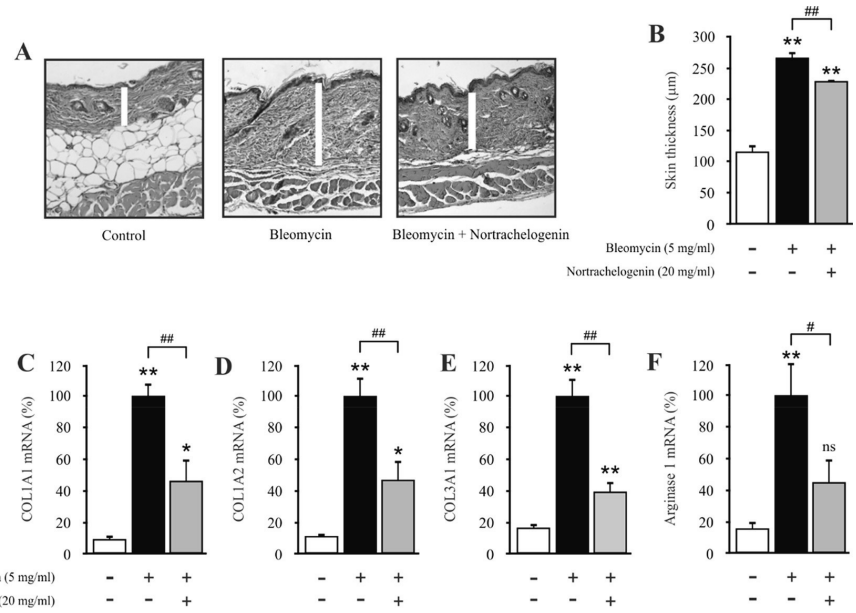

Figure 7. Effects of nortrachelogenin on bleomycin-induced dermal fibrosis. (A) Representative images of hematoxylin and eosin-stained sections of mouse skin. From left to right: control skin, skin treated with bleomycin, and skin treated with bleomycin and nortrachelogenin. (B) Quantification of dermal thickness. Dermal thickness (μm) was calculated by averaging the thickness measured at 6 locations in each section using ImageJ program. Bars represent means of the dermal thickness ± SEM; $n = 8$. (C–F) Expression of collagen type 1 alpha 1, collagen type 1 alpha 2, collagen type 3 alpha 1, and arginase 1, respectively, in control, bleomycin-treated, and bleomycin+nortrachelogenin-treated skin. mRNA levels, normalized to GAPDH, were determined with quantitative RT-PCR. Results are shown as mean ± SEM, with bleomycin alone set to 100%. $n = 7$ for bleomycin-treated skin and 8 for others. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the control. $\#p < 0.05$ and $\#\#p < 0.01$ for the indicated comparison. ns = not significant.

theoretically lead to impairment of natural anti-inflammatory and inflammation resolving processes, which may predispose to more severe symptoms and sequela of inflammatory responses. This has to be considered in the future studies on nortrachelogenin and its derivatives.

Currently, to our knowledge, there are no published data available on the human pharmacokinetics of nortrachelogenin. In mice, Yatkin and co-workers[44] demonstrated nortrachelogenin to be absorbed in an active form from perorally administered pine knot extract. Intraperitoneally administered nortrachelogenin has also been shown to be absorbed in an active form.[45] Nortrachelogenin has proved to be active in vivo, as it was found to alleviate carrageenan-induced paw edema following intraperitoneal administration.[22] The present study demonstrates biological activity of locally administered nortrachelogenin in bleomycin-induced dermal fibrosis. A possible factor confounding the current results is a potential physical/chemical interaction between bleomycin and nortrachelogenin in the injection site, possibly decreasing the activity of bleomycin. This possibility is, however, mostly ruled out by the fact that not all effects of bleomycin were reduced by nortrachelogenin treatment. Nortrachelogenin suppressed bleomycin-induced expression of collagens COL1A, COL1A2, and COL3A1 and the M2 macrophage activation marker arginase 1, while bleomycin-induced changes in iNOS, IL-13, and fibronectin were not reversed by nortrachelogenin treatment.

The detailed mechanisms of bleomycin-induced fibrosis remain mostly uncertain. Oxidative stress has been presented to contribute to the reaction, even though some recent studies have questioned this.[46] Alternatively activated macrophages are increasingly implicated in the pathogenesis of bleomycin-induced fibrosis, mimicking the situation in human fibrosing diseases.[47,48] The present findings endorse the latter mechanism. However, further studies by using other models of fibrosis such as that induced by fibrillin 1 (fbln1) tandem duplication[49] are needed to confirm the therapeutic potential of nortrachelogenin in preventing or treating aberrant fibrosis.

In the current study, arginase 1 and different collagens were measured as markers of alternative macrophage activation and fibrosis in bleomycin-exposed skin. In subsequent studies, the mechanism of nortrachelogenin could be further confirmed by measuring other markers of alternative (as well as classical) macrophage activation in the skin using immunohistochemistry. Further studies should also address possible transformations of nortrachelogenin in the body and the effects of potential bioactive metabolites produced. It should also be noted that the commercial nortrachelogenin used in the present study might contain minor amounts of those metabolites, other related compounds or other contaminating compounds, and hence the contribution of those contaminants on the observed biological effects cannot be fully ruled out. Further studies are also needed to determine the optimal mode of nortrachelogenin administration. The bioavailability of nortrachelogenin may be enhanced by, for example, developing more lipophilic derivatives or liposomal/micellar formulations, an approach that has shown efficacy with other lignans.[50]

In conclusion, our study is the first to show that nortrachelogenin, a lignan derived from *Pinus sylvestris* knot extract, can suppress alternative macrophage activation and might confer therapeutic benefits in pathological conditions

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related to aberrant alternative macrophage activation and fibrosis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b03023.

HPLC-MS/MS (MRM) chromatogram of the pine knot extract; cell viability assay of pine knot extract and nortrachelogenin; GAPDH CT values in J774 cells treated with pine knot extract or nortrachelogenin; effects of pine knot extract and nortrachelogenin on classical macrophage activation; effects of nortrachelogenin on expression of iNOS, IL-13, and fibronectin 1 in bleomycin-induced dermal fibrosis (PDF)

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■ ABBREVIATIONS USED

CCL, (C−C motif) ligand; COL, collagen; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; iNOS, inducible nitric oxide synthase; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; STAT, signal transducers and activators of transcription; T_h2 cell, type 2 helper T lymphocyte

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PUBLICATION

III

Chondrocytes from osteoarthritis patients adopt distinct phenotypes in response to central $T_H1/T_H2/T_H17$ cytokines.

Antti Pemmari, Tiina Leppänen, Mari Hämäläinen, Teemu Moilanen, Eeva Moilanen

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Article

Chondrocytes from Osteoarthritis Patients Adopt Distinct Phenotypes in Response to Central $T_H1/T_H2/T_H17$ Cytokines

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Abstract: Chronic low-grade inflammation plays a central role in the pathogenesis of osteoarthritis (OA), and several pro- and anti-inflammatory cytokines have been implicated to mediate and regulate this process. Out of these cytokines, particularly IFNγ, IL-1β, IL-4 and IL-17 are associated with different phenotypes of T helper (T_H) cells and macrophages, both examples of cells known for great phenotypic and functional heterogeneity. Chondrocytes also display various phenotypic changes during the course of arthritis. We set out to study the hypothesis of whether chondrocytes might adopt polarized phenotypes analogous to T_H cells and macrophages. We studied the effects of IFNγ, IL-1β, IL-4 and IL-17 on gene expression in OA chondrocytes with RNA-Seq. Chondrocytes were harvested from the cartilage of OA patients undergoing knee replacement surgery and then cultured with or without the cytokines for 24 h. Total RNA was isolated and sequenced, and GO (Gene Ontology) functional analysis was performed. We also separately investigated genes linked to OA in recent genome wide expression analysis (GWEA) studies. The expression of more than 2800 genes was significantly altered in chondrocytes treated with IL-1β [in the C(IL-1β) phenotype] with a fold change (FC) > 2.5 in either direction. These included a large number of genes associated with inflammation, cartilage degradation and attenuation of metabolic signaling. The profile of genes differentially affected by IFNγ (the C(IFNγ) phenotype) was relatively distinct from that of the C(IL-1β) phenotype and included several genes associated with antigen processing and presentation. The IL-17-induced C(IL-17) phenotype was characterized by the induction of a more limited set of proinflammatory factors compared to C(IL-1β) cells. The C(IL-4) phenotype induced by IL-4 displayed a differential expression of a rather small set of genes compared with control, primarily those associated with TGFβ signaling and the regulation of inflammation. In conclusion, our results show that OA chondrocytes can adopt diverse phenotypes partly analogously to T_H cells and macrophages. This phenotypic plasticity may play a role in the pathogenesis of arthritis and open new therapeutic avenues for the development of disease-modifying treatments for (osteo)arthritis.

Keywords: chondrocyte; IL-1β; IFNγ, IL-17; IL-4; RNA-Seq

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1. Introduction

Osteoarthritis (OA) is the most common form of arthritis. It has been estimated to affect up to a half of the elderly population, and therefore causes widespread disability and human suffering as well as an immense burden to healthcare systems [1]. Once thought as a mostly mechanical "wear and tear" disease, the chronic inflammatory component of osteoarthritis has been increasingly recognized during recent decades [2]. Constant low-grade inflammation in the joint contributes to pain, oxidative stress, increased catabolism, and the eventual breakdown of articular cartilage [3,4]. Despite intense research, no disease-modifying pharmacological treatments are currently available for OA [5], demonstrating that our understanding of the pathogenesis of the disease remains limited.

When comparing chondrocytes from OA patients with healthy cells, several changes in gene expression can be observed [6,7]. The potential causal roles of these changes in the pathogenesis of OA are currently largely unknown. However, some of them can be considered harmful (such as secretion of catabolic enzymes and proinflammatory cytokines) and others protective (e.g., the production of extracellular matrix [ECM] components) [8,9]. The changes in OA chondrocyte phenotype are thought to be caused by several physical and chemical factors, among them local proinflammatory cytokines [10].

The T helper (T_H) cell is probably the most well-known example of a cell capable of adopting distinct phenotypes in response to environmental factors. The different T_H phenotypes, in turn, are associated with different cytokines. The T_H1 phenotype drives inflammation and defense against intracellular pathogens. These cells are induced by interleukin 12 (IL-12) and produce mainly interferon gamma (IFNγ) as an effector cytokine [11]. In addition, they induce macrophages to produce IL-1β, which in turn promotes the proinflammatory effects of T_H1 cells [12]. T_H2 cells are induced by interleukins 2 and 4. They secrete various factors that promote humoral immunity and regulate inflammation, of which IL-4 is regarded as the central cytokine [11]. T_H17 cells are most closely associated with autoimmunity; they are induced by transforming growth factor beta (TGFβ) along with several proinflammatory cytokines, such as interleukins 6, 21 and 23, and they produce IL-17 as the central effector [13].

The macrophage is another cell type with well-defined differential phenotypes. The so-called "macrophage polarization" has two main phenotypes analogous to T_H1 and T_H2. The proinflammatory or "classically activated" M1 phenotype is associated with proinflammatory cytokines such as IL-1β and IFNγ, while the healing-promoting "alternatively activated" M2 phenotype is mainly linked to IL-4 [14]. The effects of IL-17 on macrophage phenotype have also attracted considerable interest. The M17 phenotype is not as well-defined as the M1 and M2 phenotypes; however, macrophages stimulated by IL-17 are characterized by the increased production of chemotactic and proinflammatory factors in the initial stages of the inflammatory response [15] and by the clearance of apoptotic cells and resolution of inflammation in the later phase [16].

Some authors have noted similarities between the variable functions and gene expression profiles of macrophages and chondrocytes in the setting of arthritis [17]. As another intriguing observation, major $T_H1/2/17$ cytokines have been shown to play roles in the development of different forms of arthritis. Of the cytokines that have been implicated in the development of OA, IL-1β is probably the most prominent. It has been shown to decrease the anabolic activity in chondrocytes and promote their apoptosis [18]. It also induces the expression of the proteolytic enzymes of the matrix metalloproteinase (MMP) and a disintegrin-like and metalloproteinase with trombospondin motifs (ADAMTS) families [19]. OA chondrocytes have been shown to upregulate the expression of IL-1 receptor (IL-1R) increasing their sensitivity to this cytokine [20]. Despite this, systemic treatment strategies specifically targeting IL-1β seem to have rather limited efficacy in OA [21], and none have reached clinical use.

Another major proinflammatory cytokine playing a role in the pathogenesis of arthritis is interleukin 17A (IL-17A) [22]. It promotes inflammation in concert with other proinflammatory cytokines [23], and its concentration in the synovial fluid correlates with radiographic severity of joint destruction [24]. In chondrocytes, it induces proinflammatory and catabolic factors and reduces proteoglycan synthesis [25–27]. Along with other proinflammatory cytokines, it also increases bone degradation by activating RANK ligand (RANKL) in osteoclasts [28]. In a murine model of collagen-induced arthritis, IL-17 deficiency has been shown to protect joints from the disease and IL-17 overexpression to exacerbate it [29,30]. Some functional gene expression analyses have actually implicated IL-17 signaling as a pathophysiological factor over IL-1β, the cytokine long known to drive OA [31].

In contrast to IL-1β and IL-17, the potential role of IFNγ as a causative factor in OA has attracted less interest. However, it has been found to be upregulated in chondrocytes by

proinflammatory cytokines [32] as well as to be present in OA synovial fluid [33]. Some gene variants that affect the development of OA, particularly those of T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), exert their effects via the modulation of IFNγ expression [34].

In the light of the above connections between the cytokines linked to major T helper cell/macrophage phenotypes and OA, it can be hypothesized that chondrocytes might also adopt phenotypes analogous to $T_H1/2/17$ or M1/2/17 cells, and that these phenotypes might play a role in the development of OA. In the present study, we investigated the effects of the central $T_H1/2/17$ cytokines on gene expression in OA chondrocytes. We sought to identify significantly differentially expressed genes and modulated pathways. The results were also compared to those of a recent genome-wide association study comparing degraded OA cartilage to preserved cartilage [35]. To our knowledge, this is the first study comparing the effects of the central $T_H1/2/17$ cytokines on OA chondrocytes and to characterize the resulting phenotypes.

2. Results

2.1. Effects of IL-1β on Chondrocyte Phenotype

After normalization and correction for multiple testing, a total of 2822 genes were found to be differentially expressed in IL-1β-treated chondrocytes [in the C(IL-1β) phenotype] versus controls in a statistically significant manner (FDR-corrected p-value < 0.05) and with a fold change (FC) 2.5 or more in either direction. Of these, 1092 were up- and 1730 downregulated. The list of the 20 most strongly upregulated genes contains several proinflammatory cyto- and chemokines, while the most strongly downregulated ones include several factors associated with regulation of gene expression, such as histone proteins (Table 1).

2.2. Effects of IL-17 on Chondrocyte Phenotype

Three hundred and eighty genes were differentially expressed in IL-17-treated chondrocytes [in the C(IL-17) phenotype] versus controls with FC > 2.5 in either direction, 314 of which were up- and 66 downregulated. Among the 20 most strongly upregulated genes were several associated with inflammation and chemotaxis, while the most strongly downregulated include genes involved in connective tissue development (Table 2).

2.3. Effects of IFNγ on Chondrocyte Phenotype

After normalization and correction for multiple testing, a total of 548 genes were found to be differentially expressed in IFNγ-treated chondrocytes [in the C(IFNγ) phenotype] versus controls in a statistically significant manner and FC 2.5 or more in either direction. Of these, 462 were up- and 86 downregulated. The 20 genes most strongly upregulated in C(IFNγ) cells included many associated with inflammation, antigen processing and presentation, and the regulation of proliferation. The most strongly downregulated genes included those involved in cell adhesion, proliferation and migration, and in Wnt signaling (Table 3).

2.4. Effects of IL-4 on Chondrocyte Phenotype

Twenty-six genes were upregulated by IL-4 with FC > 2.5 (Table S1). No genes were downregulated by IL-4 to a similar extent, but 10 genes were downregulated with FC < −1.5 (Table S2). In the C(IL-4) phenotype, the upregulated genes included those associated with the regulation of inflammation and TGFβ signaling as well as metabolism and cell adhesion, while several genes linked to cell proliferation were among the downregulated ones.

Table 1. Twenty most strongly up- and downregulated genes in interleukin 1-treated OA chondrocytes (IL1) relative to control (Co).

Gene	Name	Function	Mean (Co)	Mean (IL1)	Fold Change	adj. p
IL6	Interleukin 6	Inflammation	12.4	18,406.9	**3685.72**	$<1.0 \times 10^{-4}$
CXCL1	C-X-C motif chemokine ligand 1	Inflammation, chemotaxis	13.8	23,793.7	**3457.68**	$<1.0 \times 10^{-4}$
IL1B	Interleukin 1 beta	Inflammation	2.8	9575.7	**3332.44**	$<1.0 \times 10^{-4}$
CXCL8	C-X-C motif chemokine ligand 8	Inflammation, chemotaxis	329.5	855,146.3	**2968.9**	$<1.0 \times 10^{-4}$
CXCL6	C-X-C motif chemokine ligand 6	Inflammation, chemotaxis	2.8	4951.8	**2352.02**	$<1.0 \times 10^{-4}$
CXCL5	C-X-C motif chemokine ligand 5	Inflammation, chemotaxis	7.4	7352.4	**1239.8**	$<1.0 \times 10^{-4}$
CXCL2	C-X-C motif chemokine ligand 2	Inflammation, chemotaxis	3.9	4798.2	**1198.05**	$<1.0 \times 10^{-4}$
CXCL3	C-X-C motif chemokine ligand 3	Inflammation, chemotaxis	3.1	3154.6	**1130.76**	$<1.0 \times 10^{-4}$
CCL20	C-C motif chemokine ligand 20	Inflammation, chemotaxis	418	381,100.8	**1128.35**	$<1.0 \times 10^{-4}$
IL36RN	Interleukin 36 receptor antagonist	Regulation of inflammation	8.6	5863.8	**914.19**	$<1.0 \times 10^{-4}$
ADORA2A	Adenosine A2a receptor	Regulation of inflammation	5.5	1550.7	**641.44**	$<1.0 \times 10^{-4}$
IL36G	Interleukin 36 gamma	Inflammation	1.8	1065.5	**562.03**	$<1.0 \times 10^{-4}$
EREG	Epiregulin	Regulation of proliferation	31.9	13,697.7	**506.87**	$<1.0 \times 10^{-4}$
CSF3	Colony stimulating factor 3	Granulocyte-mediated inflammation	0.1	63.9	**300.02**	$<1.0 \times 10^{-4}$
VNN1	Vanin 1	T cell migration	9.2	2467.2	**273.35**	$<1.0 \times 10^{-4}$
CCL5	C-C motif chemokine ligand 5	Inflammation, chemotaxis	4.1	1134.2	**271.85**	$<1.0 \times 10^{-4}$
C15orf48	Chromosome 15 open reading frame 48	?	27.2	4669.1	**253.13**	$<1.0 \times 10^{-4}$
CCL3	C-C motif chemokine ligand 3	Inflammation, granulocyte activation	0.5	166.3	**242.88**	$<1.0 \times 10^{-4}$
FCAMR	Fc fragment of IgA and IgM receptor	Adaptive immunity, leukocyte migration	2.6	492	**213.45**	$<1.0 \times 10^{-4}$
SERPINB7	Serpin family B member 7	Endoproteinase inhibition	22.1	3747.9	**205.63**	$<1.0 \times 10^{-4}$
HRCT1	Histidine rich carboxyl terminus 1	?	105.8	4.1	**−38.85**	$<1.0 \times 10^{-4}$
LSP1	Lymphocyte specific protein 1	Regulation of neutrophil mobility	1749.6	58.1	**−31.39**	$<1.0 \times 10^{-4}$
HIST1H3G	Histone cluster 1 H3 family member g	Regulation of transcription	183.4	9.6	**−28.26**	$<1.0 \times 10^{-4}$
ACTC1	Actin, alpha, cardiac muscle 1	Heart muscle constituent	195.2	10.5	**−24.79**	$<1.0 \times 10^{-4}$
NXPH3	Neurexophilin 3	?	39.2	2.4	**−23.89**	$<1.0 \times 10^{-4}$
SCN2B	Sodium voltage-gated channel beta subunit 2	Cell adhesion and migration	167	8.7	**−22.19**	$<1.0 \times 10^{-4}$
HIST1H1A	Histone cluster 1 H1 family member a	?	908.5	47.2	**−21.2**	$<1.0 \times 10^{-4}$
GDF10	Growth differentiation factor 10	Skeletal system development	813.6	45.7	**−20.57**	$<1.0 \times 10^{-4}$
LINC02593	Long intergenic non-protein coding RNA 2593	?	68.3	3.4	**−20.53**	$<1.0 \times 10^{-4}$
HIST1H3B	Histone cluster 1 H3 family member b	Regulation of transcription	990.6	59.2	**−20.46**	$<1.0 \times 10^{-4}$
TMEM26	Transmembrane protein 26	?	403.7	21.4	**−19.3**	$<1.0 \times 10^{-4}$
PHYHIPL	Phytanoyl-CoA 2-hydroxylase interacting protein like	?	22	1.6	**−19.19**	$<1.0 \times 10^{-4}$
SARDH	Sarcosine dehydrogenase	Mitochondrial metabolism	25.8	2.4	**−19.08**	$<1.0 \times 10^{-4}$
HIST1H2BO	Histone cluster 1 H2B family member o	Regulation of transcription?	234.4	12.7	**−18.99**	$<1.0 \times 10^{-4}$

Table 1. *Cont.*

Gene	Name	Function	Mean (Co)	Mean (IL1)	Fold Change	adj. p
ID3	Inhibitor of DNA binding 3, HLH protein	Regulation of transcription	676.5	45.8	**−18.32**	$<1.0 \times 10^{-4}$
HIST1H2AJ	Histone cluster 1 H2A family member j	Regulation of transcription?	857	47.1	**−18.12**	$<1.0 \times 10^{-4}$
HIST1H1B	Histone cluster 1 H1 family member b	Regulation of transcription?	736	50.6	**−17.69**	$<1.0 \times 10^{-4}$
MFAP2	Microfibril associated protein 2	ECM organization	33	3.2	**−17.52**	$<1.0 \times 10^{-4}$
TNNT3	Troponin T3, fast skeletal type	Muscle constituent	95.6	6.4	**−17.51**	$<1.0 \times 10^{-4}$
HIST1H2AL	Histone cluster 1 H2A family member l	Regulation of transcription?	321.4	21.2	**−17.32**	$<1.0 \times 10^{-4}$

Red = upregulated genes; blue = downregulated genes.

Table 2. Twenty most strongly up- and downregulated genes in interleukin 17-treated OA chondrocytes (IL17) relative to control (Co).

Gene	Name	Function	Mean (Co)	Mean (IL17)	Fold Change	adj. p
SAA2	Serum amyloid A2	Chemotaxis	5.5	659.2	**319.99**	$<1.0 \times 10^{-4}$
IL6	Interleukin 6	Inflammation	12.2	1431.4	**250.15**	$<1.0 \times 10^{-4}$
SAA1	Serum amyloid A1	Inflammation, chemotaxis	63.7	3520.0	**183.26**	$<1.0 \times 10^{-4}$
SAA2-SAA4	SAA2-SAA4 readthrough	Chemotaxis?	2.9	216.7	**156.18**	$<1.0 \times 10^{-4}$
CXCL6	C-X-C motif chemokine ligand 6	Inflammation, chemotaxis	2.8	276.4	**141.01**	$<1.0 \times 10^{-4}$
CXCL1	C-X-C motif chemokine ligand 1	Inflammation, chemotaxis	13.6	1170.5	**136.48**	$<1.0 \times 10^{-4}$
VNN1	Vanin 1	T cell migration	9.1	820.5	**84.13**	$<1.0 \times 10^{-4}$
CCL20	C-C motif chemokine ligand 20	Chemotaxis	412.8	26,508.9	**73.49**	$<1.0 \times 10^{-4}$
TNFSF18	TNF superfamily member 18	T cell survival	4.2	470.3	**73.05**	$<1.0 \times 10^{-4}$
IL36RN	Interleukin 36 receptor antagonist	Regulation of inflammation	8.5	468.0	**69.09**	$<1.0 \times 10^{-4}$
VNN3	Vanin 3	?	1.8	130.3	**66.35**	$<1.0 \times 10^{-4}$
ADORA2A	Adenosine A2a receptor	Inflammation, phagocytosis	5.4	105.9	**64.74**	$<1.0 \times 10^{-4}$
CXCL2	C-X-C motif chemokine ligand 2	Inflammation, chemotaxis	3.9	220.3	**55.90**	$<1.0 \times 10^{-4}$
CXCL8	C-X-C motif chemokine ligand 8	Inflammation, chemotaxis	324.8	14,116.5	**48.18**	$<1.0 \times 10^{-4}$
C15orf48	Chromosome 15 open reading frame 48	Mitochondrial respiration?	26.9	820.3	**46.34**	$<1.0 \times 10^{-4}$
PDZK1IP1	PDZK1 interacting protein 1	Regulation of apoptosis	5.2	206.9	**41.18**	$<1.0 \times 10^{-4}$
NOS2	Nitric oxide synthase 2	Inflammation	137.9	3370.2	**40.02**	$<1.0 \times 10^{-4}$
ODAPH	Odontogenesis associated phosphoprotein	Enamel production	1.4	41.9	**37.29**	$<1.0 \times 10^{-4}$
SLC28A3	Solute carrier family 28 member 3	Nucleoside transport	4.3	150.4	**35.34**	$<1.0 \times 10^{-4}$
CXCL5	C-X-C motif chemokine ligand 5	Inflammation, chemotaxis	7.3	207.5	**34.25**	$<1.0 \times 10^{-4}$

Table 2. *Cont.*

Gene	Name	Function	Mean (Co)	Mean (IL17)	Fold Change	adj. p
ACTC1	Actin, alpha, cardiac muscle 1	Cardiac muscle component	191.7	26.7	**−8.14**	$<1.0 \times 10^{-4}$
TOX	Thymocyte selection associated high mobility group box	T cell development	14.6	3.9	**−5.66**	0.0010
TMEM26	Transmembrane protein 26	?	396.3	69.8	**−5.47**	$<1.0 \times 10^{-4}$
TNNT3	Troponin T3, fast skeletal type	Muscle component	93.9	17.9	**−5.28**	$<1.0 \times 10^{-4}$
TENT5B	Terminal nucleotidyltransferase 5B	Regulation of cell proliferation	152.5	39.7	**−4.81**	$<1.0 \times 10^{-4}$
TMEM26-AS1	TMEM26 antisense RNA 1	?	32.0	14.4	**−4.77**	3.8×10^{-4}
RCAN2	Regulator of calcineurin 2	Regulation of transcription	326.5	74.6	**−4.74**	$<1.0 \times 10^{-4}$
OPRL1	Opioid related nociceptin receptor 1	?	11.8	3.0	**−4.51**	0.0068
CSRNP3	Cysteine and serine rich nuclear protein 3	Regulation of apoptosis	59.7	19.7	**−4.01**	$<1.0 \times 10^{-4}$
ASPN	Asporin	Cartilage constituent	2011.2	505.2	**−3.92**	$<1.0 \times 10^{-4}$
HRCT1	Histidine rich carboxyl terminus 1	?	104.1	25.8	**−3.85**	$<1.0 \times 10-4$
AQP1	Aquaporin 1 (Colton blood group)	Regulation of osmotic pressure, angiogenesis, apoptosis	42.9	13.4	**−3.69**	$<1.0 \times 10^{-4}$
YWHAZP5	YWHAZ pseudogene 5	?	10.2	3.2	**−3.68**	0.013
MRAP2	Melanocortin 2 receptor accessory protein 2	cAMP signaling	1295.9	376.5	**−3.62**	$<1.0 \times 10^{-4}$
C1QTNF7	C1q and TNF related 7	?	63.4	20.1	**−3.54**	$<1.0 \times 10^{-4}$
MFAP2	Microfibril associated protein 2	Connective tissue organization	32.4	8.7	**−3.47**	$<1.0 \times 10^{-4}$
CLEC3A	C-type lectin domain family 3 member A	Skeletal system development	847.3	264.6	**−3.46**	$<1.0 \times 10^{-4}$
GREM1	Gremlin 1, DAN family BMP antagonist	Regulation of connective tissue development	5141.6	1566.4	**−3.41**	$<1.0 \times 10^{-4}$
CRISPLD1	Cysteine rich secretory protein LCCL domain containing 1	Morphogenesis	946.1	280.2	**−3.39**	$<1.0 \times 10^{-4}$
HRASLS5 (=PLAAT5)	HRAS like suppressor family member 5	Glycerophospholipid metabolism	12.8	3.6	**−3.37**	0.019

Red = upregulated genes; blue = downregulated genes.

Table 3. Twenty most strongly up- and downregulated genes in interferon gamma -treated OA chondrocytes (IFNγ) relative to control (Co).

Gene	Name	Function	Mean (Co)	Mean (IFNγ)	Fold change	adj. p
IDO1	Indoleamine 2,3-dioxygenase 1	Regulation of T cell -mediated immunity	17.5	42,320.0	**4643.74**	$<1.0 \times 10^{-4}$
LGALS17A	Galectin 14 pseudogene	?	0.4	1065.1	**1750.58**	$<1.0 \times 10^{-4}$
GBP1P1	Guanylate binding protein 1 pseudogene 1	?	2.6	2838.8	**1245.34**	$<1.0 \times 10^{-4}$
CXCL10	C-X-C motif chemokine ligand 10	Chemotaxis	2.2	2065.2	**1117.91**	$<1.0 \times 10^{-4}$
GBP5	Guanylate binding protein 5	Inflammasome activation	1.4	1518.3	**1112.44**	$<1.0 \times 10^{-4}$
CXCL9	C-X-C motif chemokine ligand 9	T cell chemotaxis	1.1	1069.9	**1033.80**	$<1.0 \times 10^{-4}$
GBP4	Guanylate binding protein 4	Inflammation?	30.9	27,565.6	**955.57**	$<1.0 \times 10^{-4}$

Table 3. *Cont.*

Gene	Name	Function	Mean (Co)	Mean (IFNγ)	Fold change	adj. p
IFI44L	Interferon induced protein 44 like	?	9.7	6185.8	**694.66**	$<1.0 \times 10^{-4}$
GBP1	Guanylate binding protein 1	Negative regulation of inflammation	124.3	54,562.1	**454.62**	$<1.0 \times 10^{-4}$
HLA-DRA	Major histocompatibility complex, class II, DR alpha	Antigen presentation	5.6	2338.3	**408.93**	$<1.0 \times 10^{-4}$
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	Antigen presentation	10.7	2430.7	**383.18**	$<1.0 \times 10^{-4}$
CD74	CD74 molecule	Antigen presentation	31.9	11,211.5	**353.35**	$<1.0 \times 10^{-4}$
RSAD2	Radical S-adenosyl methionine domain containing 2	Antiviral action	44.5	15,365.2	**338.82**	$<1.0 \times 10^{-4}$
RARRES3	Retinoic acid receptor responder 3	Phospholipid catabolism	33.1	8271.1	**286.40**	$<1.0 \times 10^{-4}$
BST2	Bone marrow stromal cell antigen 2	Antiviral action	10.1	2908.5	**285.04**	$<1.0 \times 10^{-4}$
GBP6	Guanylate binding protein family member 6	Inflammation	1.0	193.3	**273.26**	$<1.0 \times 10^{-4}$
HLA-DRB5	Major histocompatibility complex, class II, DR beta 5	Antigen presentation	4.4	825.4	**253.47**	$<1.0 \times 10^{-4}$
HLA-DRB6	Major histocompatibility complex, class II, DR beta 6 (pseudogene)	Antigen presentation?	0.3	125.7	**226.68**	$<1.0 \times 10^{-4}$
APOL4	Apolipoprotein L4	Lipid metabolism	2.6	500.8	**225.95**	$<1.0 \times 10^{-4}$
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	Regulation of proliferation	96.2	20,648.8	**225.79**	$<1.0 \times 10^{-4}$
TNFRSF10D	TNF receptor superfamily member 10d	Inhibition of apoptosis	4135.1	501.9	**−7.65**	$<1.0 \times 10^{-4}$
ARHGAP9	Rho gtpase activating protein 9	?	10.7	2.4	**−5.27**	0.0028
NANOS1	Nanos C2HC-type zinc finger 1	Regulation of translation and cell migration	83.4	16.9	**−4.94**	$<1.0 \times 10^{-4}$
SNORD108	Small nucleolar RNA, C/D box 108	?	66.6	13.8	**−4.81**	$<1.0 \times 10^{-4}$
FAM189A2	Family with sequence similarity 189 member A2	?	13.6	4.3	**−4.39**	0.0033
PWAR6	Prader Willi/Angelman region RNA 6	?	34.0	7.9	**−4.32**	$<1.0 \times 10^{-4}$
GABRA4	Gamma-aminobutyric acid type A receptor alpha4 subunit	Synaptic transmission	2346.1	549.2	**−4.28**	$<1.0 \times 10^{-4}$
CORO2A	Coronin 2A	?	13.5	3.7	**−4.11**	0.020
WFDC1	WAP four-disulfide core domain 1	Regulation of proliferation	65.1	18.0	**−4.06**	$<1.0 \times 10^{-4}$
PRSS35	Serine protease 35	?	51.4	13.5	**−4.01**	$<1.0 \times 10^{-4}$
SLC16A14	Solute carrier family 16 member 14	Organic acid transport	40.2	13.3	**−3.98**	$<1.0 \times 10^{-4}$
PWAR5	Prader Willi/Angelman region RNA 5	?	359.7	91.4	**−3.93**	$<1.0 \times 10^{-4}$
MTURN	Maturin, neural progenitor differentiation regulator homolog	?	1857.1	519.7	**−3.63**	$<1.0 \times 10^{-4}$
C1QTNF5	C1q and TNF related 5	Cell adhesion	152.4	46.1	**−3.47**	$<1.0 \times 10^{-4}$
LONRF2	LON peptidase N-terminal domain and ring finger 2	?	206.8	59.5	**−3.46**	$<1.0 \times 10^{-4}$
FGFR4	Fibroblast growth factor receptor 4	Cell proliferation and migration	11.1	5.1	**−3.31**	0.045
TRABD2B	Trab domain containing 2B	Wnt signaling, proteolysis	14.2	5.5	**−3.29**	0.0014
TNNT3	Troponin T3, fast skeletal type	Muscle contraction	106.0	31.6	**−3.26**	$<1.0 \times 10^{-4}$
NCALD	Neurocalcin delta	Endocytosis	17.3	6.6	**−3.24**	0.029
CDH2	Cadherin 2	Cell adhesion	12.0	4.1	**−3.23**	0.0012

Red = upregulated genes; blue = downregulated genes.

2.5. Functional Gene Categories in Different Chondrocyte Phenotypes

Table 4 shows the Gene Ontology (GO) terms affected with a high significance (FDR-corrected p-value < 0.01) by at least one studied proinflammatory cytokine (IL-1β, IFNγ or IL-17). The C(IL-1β) phenotype was involved in the activation of a wide range of inflammatory terms and pathways, along with those related to cell adhesion as well as extracellular matrix production and degradation. The T_H17-associated cytokine IL-17 affected a partly overlapping, but smaller, set of inflammatory cytokines compared to IL-1β. The C(IFNγ) phenotype was quite distinct compared to the C(IL-1β) and C(IL-17) phenotypes; several terms related to antigen processing and presentation were affected by this cytokine alone. Nitric oxide synthase biosynthetic process and chemotaxis were among the functions involved solely in the C(IL-17) phenotype. In addition, many high-level GO terms related to inflammation were affected by all of the three proinflammatory cytokines.

In C(IL-4) cells, no significantly affected GO terms were detected when analyzing the genes with FC > 2.5 in either direction. When the FC threshold was lowered to 1.5, GO terms associated with cell division were among the significant ones (Table S3).

2.6. Comparing the Effects of Different Proinflammatory Cytokines

Next, we cross-compared the genes markedly upregulated (FC > 2.5) in the C(IL-1β), C(IFNγ) and C(IL-17) phenotypes to further characterize the differences and similarities between the resulting phenotypes. As shown in Figure 1A, a large portion (nearly 85%) of genes markedly upregulated in C(IL-17) cells were included in the large set of those similarly affected by IL-1β, but 45 genes were solely affected by IL-17, and the overlap of C(IL-17) and C(IFNγ) phenotypes was considerable smaller than that of C(IL-17) and C(IL-1β). The intersection of genes upregulated by both IL-17 and IFNγ was nearly completely contained in those upregulated by IL-1β (Figure 1A). Many central regulators of inflammation such as *IL6*, *PTGS2* (cyclo-oxygenase 2 or COX-2) and *NOS2* (inducible nitric oxide synthase or iNOS) were markedly upregulated by all the three T_H1/T_H17 cytokines, in line with the widespread activation of inflammatory pathways observed in the GO analysis (Table 5).

When comparing genes markedly downregulated (FC < −2.5) by the three proinflammatory cytokines, the large (>1000 genes) list of genes downregulated by IL-1β again contained a large proportion (85%) of those downregulated by IL-17 and a smaller amount (48%) of genes similarly affected by IFNγ (Figure 1B). Genes downregulated by all of the three cytokines are presented in Table 6 and include, for example, those associated with cell proliferation and skeletal system development.

2.7. Effects of the Cytokines on Genes Differentially Expressed in Degraded and Preserved OA Cartilage

Some previous studies have investigated the differences in gene expression between degraded and preserved OA cartilage. Of these, the study by Almeida et al. [35] is probably the most comprehensive. To see whether the studied cytokines shift chondrocyte phenotype towards either degraded or preserved cartilage, we compared the differentially expressed genes in the phenotypes observed in the present study to those differentially expressed in the study by Almeida et al. [35] As a very large number (over 2300) of significantly differentially expressed genes were identified in that study, we focused on those 84 genes which were most strongly upregulated (FC > 2.5 and FDR-corrected p-value < 0.01) in the degraded cartilage. Of those 84 genes, 38 were significantly affected by at least one of the proinflammatory cytokines (IL-1, IL-17 or IFNγ) in our data. A large majority (30) of these 38 genes were also upregulated by IL-1β, showing that the cytokine shifts chondrocyte phenotype towards the one observed in the degraded cartilage. Several mediators of inflammation, such as *LIF*, *CCL20* and *TREM1*, were especially strongly upregulated. Only four of the 84 genes (namely *CLIC3*, *ERFE*, *SLC27A2* and *ANK3*) were downregulated by IL-1β.

Table 4. GO terms affected by different proinflammatory cytokines. Genes with FC > 2.5 in either direction were analyzed with DAVID, and the resulting lists were reduced with REVIGO. GO terms significantly affected (with FDR-corrected *p*-value < 0.05) by a cytokine are marked with an X.

Term	IL1	IL17	IFNγ	Term	IL1	IL17	IFNγ
Inflammatory response	X	X	X	Nucleosome assembly	X		
Immune response	X	X	X	Chromosome segregation	X		
Response to lipopolysaccharide	X	X	X	Protein heterotetramerization	X		
Chemotaxis	X	X	X	Wound healing	X		
Negative regulation of viral entry into host cell	X	X	X	Regulation of cell proliferation	X		
				Cell migration	X		
Negative regulation of type I interferon production	X	X	X	Regulation of gene silencing	X		
				Positive regulation of interleukin-12 production	X		
Response to progesterone	X	X		Odontogenesis	X		
Cell-cell signaling	X	X		Cellular response to mechanical stimulus	X		
Angiogenesis	X	X		Peptidyl-tyrosine phosphorylation	X		
Negative regulation of growth	X	X		Collagen catabolic process	X		
Positive regulation of mitotic nuclear division	X	X		Positive regulation of cell division	X		
				Positive chemotaxis		X	
Negative regulation of cell proliferation	X	X		Positive regulation of nitric-oxide synthase biosynthetic process		X	
Signal transduction	X		X	Acute-phase response		X	
Response to virus	X		X	Positive regulation of cytosolic calcium ion concentration		X	
Positive regulation of interleukin-6 production	X		X	Positive regulation of gtpase activity			X
				Response to glucocorticoid			X
Response to hydrogen peroxide	X		X	Response to wounding			X
Positive regulation of I-kappab kinase/NF-kappab signaling	X		X	Positive regulation of NF-kappab transcription factor activity			X
Response to drug	X		X	Negative regulation of tumor necrosis factor production			X
Cellular response to zinc ion		X	X	Cellular response to organic cyclic compound			X
Response to toxic substance		X	X	Antigen processing and presentation			X
Tumor necrosis factor-mediated signaling pathway		X	X	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II			X
Cell division	X			Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent			X
DNA replication	X						
Telomere organization	X			Response to interferon-beta			X
Positive regulation of gene expression	X			Response to interferon-alpha			X
				T cell costimulation			X
Cell adhesion	X			Positive regulation of T cell mediated cytotoxicity			X
Extracellular matrix organization	X			Defense response			X
Skeletal system development	X			Protein trimerization			X
Sister chromatid cohesion	X			Proteolysis			X
DNA replication initiation	X			Defense response to protozoan			X
Cellular protein metabolic process	X			Positive regulation of peptidyl-tyrosine phosphorylation			X
Cell proliferation	X			Protein polyubiquitination			X
Negative regulation of gene expression, epigenetic	X						

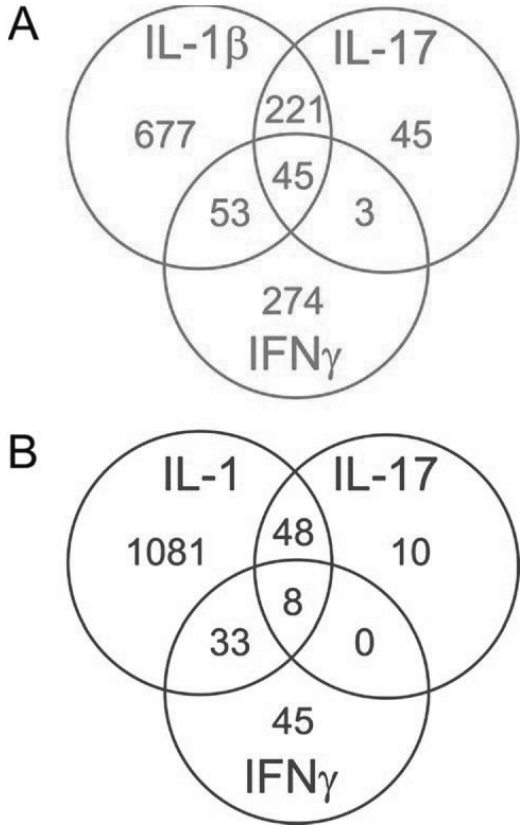

Figure 1. Venn diagrams of genes markedly upregulated (FC > 2.5) (**A**) or markedly downregulated (FC < 2.5) (**B**) by IL-1β, IL-17 and IFNγ. Red denotes up- and blue downregulated genes.

In the C(IFNγ) phenotype, 13 of the 84 genes associated with degraded cartilage (including *LIF* and *NGF*) were upregulated compared with control, but nearly as many (nine) were downregulated, including *TREM1*. This shows that the effects of IFNγ on chondrocyte phenotype in relation to the degraded/preserved cartilage are more ambiguous than those of IL-1β.

In C(IL-17) chondrocytes, 25 of the 84 genes associated with degraded cartilage were upregulated compared to naïve chondrocytes (including *CCL20* and *IL11*), and none were significantly downregulated. Nine genes, including *IGFBP1*, *LIF* and *GPR158*, were upregulated in all three inflammatory phenotypes C(IL-1β), C(IFNγ) and C(IL-17) and one (*ANK3*) was downregulated in all of them. (Figure 2 and Table S4).

Table 5. Genes upregulated by all studied proinflammatory cytokines with FC > 2.5. Shown are mean normalized expression levels in control (Co) and in C(IL1), C(IL17) and C(IFNγ) phenotypes, fold changes (FCs) for all comparisons vs. control and false discovery rate (FDR)-adjusted *p* values for them.

Gene	Name	Mean exp. (Co)	Mean exp. (IL1)	Mean exp. (IL17)	Mean exp. (IFNγ)	FC (IL1 vs. Co)	adj. p (IL1 vs. Co)	FC (IL17 vs. Co)	adj. p (IL17 vs. Co)	FC (IFNγ vs. Co)	adj. p (IFNγ vs. Co)
IL6	Interleukin 6	12.8	18,406.9	1431.4	94.2	**3685.72**	$<1.0 \times 10^{-4}$	**250.15**	$<1.0 \times 10^{-4}$	**12.34**	$<1.0 \times 10^{-4}$
IL36RN	Interleukin 36 receptor antagonist	8.9	5863.8	468.0	36.7	**914.19**	$<1.0 \times 10^{-4}$	**69.09**	$<1.0 \times 10^{-4}$	**4.59**	$<1.0 \times 10^{-4}$
ESM1	Endothelial cell specific molecule 1	276.7	37,984.1	1373.5	1449.2	**157.25**	$<1.0 \times 10^{-4}$	**5.09**	$<1.0 \times 10^{-4}$	**4.70**	$<1.0 \times 10^{-4}$
SAA2	Serum amyloid A2	5.8	371.4	659.2	27.1	**149.11**	$<1.0 \times 10^{-4}$	**319.99**	$<1.0 \times 10^{-4}$	**8.73**	$<1.0 \times 10^{-4}$
iNOS/NOS2	Inducible nitric oxide synthase/Nitric oxide synthase 2	144.2	12,704.9	3370.2	3046.1	**131.22**	$<1.0 \times 10^{-4}$	**40.02**	$<1.0 \times 10^{-4}$	**30.16**	$<1.0 \times 10^{-4}$
NOD2	Nucleotide binding oligomerization domain containing 2	7.6	919.4	96.7	43.9	**116.73**	$<1.0 \times 10^{-4}$	**13.67**	$<1.0 \times 10^{-4}$	**5.61**	$<1.0 \times 10^{-4}$
PTX3	Pentraxin 3	184.4	18,888.7	4615.3	479.6	**113.19**	$<1.0 \times 10^{-4}$	**27.47**	$<1.0 \times 10^{-4}$	**2.60**	$<1.0 \times 10^{-4}$
SAA1	Serum amyloid A1	66.6	2188.7	3520.0	227.6	**94.66**	$<1.0 \times 10^{-4}$	**183.26**	$<1.0 \times 10^{-4}$	**6.46**	$<1.0 \times 10^{-4}$
CD300E	CD300e molecule	3.6	316.9	32.7	71.6	**72.79**	$<1.0 \times 10^{-4}$	**7.91**	$<1.0 \times 10^{-4}$	**17.15**	$<1.0 \times 10^{-4}$
IL36B	Interleukin 36 beta	11.3	466.3	80.1	39.1	**67.27**	$<1.0 \times 10^{-4}$	**9.65**	$<1.0 \times 10^{-4}$	**3.60**	$<1.0 \times 10^{-4}$
TNFRSF1B	TNF receptor superfamily member 1B	40.0	2370.7	525.8	118.9	**62.58**	$<1.0 \times 10^{-4}$	**14.66**	$<1.0 \times 10^{-4}$	**3.02**	$<1.0 \times 10^{-4}$
TNFAIP6	TNF alpha induced protein 6	1176.4	42,950.3	5512.4	4561.2	**36.87**	$<1.0 \times 10^{-4}$	**4.59**	$<1.0 \times 10^{-4}$	**3.59**	$<1.0 \times 10^{-4}$
TMEM132A	Transmembrane protein 132A	10.3	328.1	165.0	32.6	**33.90**	$<1.0 \times 10^{-4}$	**16.64**	$<1.0 \times 10^{-4}$	**3.18**	$<1.0 \times 10^{-4}$
ICAM1	Intercellular adhesion molecule 1	1415.2	42,657.2	4388.3	8524.5	**31.66**	$<1.0 \times 10^{-4}$	**3.15**	$<1.0 \times 10^{-4}$	**5.54**	$<1.0 \times 10^{-4}$
C3AR1	Complement C3a receptor 1	2.2	66.2	11.4	11.2	**28.15**	$<1.0 \times 10^{-4}$	**6.36**	1.5×10^{-4}	**5.32**	4.9×10^{-4}
CLEC2B	C-type lectin domain family 2 member B	5.3	145.0	48.5	20.6	**27.53**	$<1.0 \times 10^{-4}$	**9.35**	$<1.0 \times 10^{-4}$	**3.85**	$<1.0 \times 10^{-4}$
COX-2/PTGS2	Cyclooxygenase-2/Prostaglandin-endoperoxide synthase 2	1310.7	37,281.5	4678.6	5349.2	**26.96**	$<1.0 \times 10^{-4}$	**3.28**	$<1.0 \times 10^{-4}$	**3.57**	$<1.0 \times 10^{-4}$
TLR2	Toll like receptor 2	134.9	3348.9	782.0	371.4	**22.64**	$<1.0 \times 10^{-4}$	**5.02**	$<1.0 \times 10^{-4}$	**2.54**	$<1.0 \times 10^{-4}$
CCL7	C-C motif chemokine ligand 7	2.1	36.7	20.6	24.4	**20.66**	$<1.0 \times 10^{-4}$	**12.14**	$<1.0 \times 10^{-4}$	**10.56**	$<1.0 \times 10^{-4}$
CCL2	C-C motif chemokine ligand 2	150.4	2475.0	815.0	430.6	**19.42**	$<1.0 \times 10^{-4}$	**5.85**	$<1.0 \times 10^{-4}$	**2.61**	$<1.0 \times 10^{-4}$
IRF4	Interferon regulatory factor 4	23.5	400.1	94.9	114.2	**18.20**	$<1.0 \times 10^{-4}$	**4.62**	$<1.0 \times 10^{-4}$	**4.69**	$<1.0 \times 10^{-4}$
CD274	CD274 molecule	61.8	1048.8	350.1	3845.7	**17.56**	$<1.0 \times 10^{-4}$	**6.18**	$<1.0 \times 10^{-4}$	**60.08**	$<1.0 \times 10^{-4}$
RBM47	RNA binding motif protein 47	8.8	122.3	30.6	22.8	**14.96**	$<1.0 \times 10^{-4}$	**3.38**	$<1.0 \times 10^{-4}$	**2.67**	0.040
CD38	CD38 molecule	9.8	133.8	74.3	211.4	**14.81**	$<1.0 \times 10^{-4}$	**7.67**	$<1.0 \times 10^{-4}$	**20.76**	$<1.0 \times 10^{-4}$
BDKRB1	Bradykinin receptor B1	29.0	401.5	129.6	105.0	**13.95**	$<1.0 \times 10^{-4}$	**4.88**	$<1.0 \times 10^{-4}$	**3.19**	$<1.0 \times 10^{-4}$
GCH1	GTP cyclohydrolase 1	591.7	7968.7	2212.7	3584.2	**13.38**	$<1.0 \times 10^{-4}$	**3.90**	$<1.0 \times 10^{-4}$	**5.63**	$<1.0 \times 10^{-4}$
LRRC38	Leucine rich repeat containing 38	11.2	132.1	44.4	35.8	**11.59**	$<1.0 \times 10^{-4}$	**3.79**	$<1.0 \times 10^{-4}$	**2.98**	$<1.0 \times 10^{-4}$
KIAA1217	KIAA1217	15.3	157.8	55.1	109.1	**10.61**	$<1.0 \times 10^{-4}$	**3.80**	$<1.0 \times 10^{-4}$	**6.39**	$<1.0 \times 10^{-4}$
SSTR2	Somatostatin receptor 2	90.0	971.2	1549.7	340.1	**10.56**	$<1.0 \times 10^{-4}$	**16.11**	$<1.0 \times 10^{-4}$	**3.36**	$<1.0 \times 10^{-4}$
DUSP5	Dual specificity phosphatase 5	77.3	746.8	302.4	236.1	**10.54**	$<1.0 \times 10^{-4}$	**4.02**	$<1.0 \times 10^{-4}$	**2.90**	$<1.0 \times 10^{-4}$
TYMP	Thymidine phosphorylase	311.3	3020.1	1275.1	9324.0	**10.15**	$<1.0 \times 10^{-4}$	**4.24**	$<1.0 \times 10^{-4}$	**28.71**	$<1.0 \times 10^{-4}$
GPR158	G protein-coupled receptor 158	6.9	38.0	22.0	21.5	**9.98**	$<1.0 \times 10^{-4}$	**6.77**	0.0018	**5.55**	7.6×10^{-4}
PRLR	Prolactin receptor	8.3	78.8	29.7	33.0	**9.93**	$<1.0 \times 10^{-4}$	**3.05**	0.0034	**3.92**	$<1.0 \times 10^{-4}$
GSAP	Gamma-secretase activating protein	122.2	1109.8	378.0	509.3	**9.18**	$<1.0 \times 10^{-4}$	**3.26**	$<1.0 \times 10^{-4}$	**3.74**	$<1.0 \times 10^{-4}$

Table 5. *Cont.*

Gene	Name	Mean exp. (Co)	Mean exp. (IL1)	Mean exp. (IL17)	Mean exp. (IFNγ)	FC (IL1 vs. Co)	adj. p (IL1 vs. Co)	FC (IL17 vs. Co)	adj. p (IL17 vs. Co)	FC (IFNγ vs. Co)	adj. p (IFNγ vs. Co)
GPR39	G protein-coupled receptor 39	15.4	110.6	39.1	41.4	**9.17**	$<1.0 \times 10^{-4}$	**3.24**	1.7×10^{-4}	**2.71**	$<1.0 \times 10^{-4}$
LYPD1	LY6/PLAUR domain containing 1	10.5	71.5	28.7	27.7	**8.44**	$<1.0 \times 10^{-4}$	**3.31**	5.6×10^{-4}	**2.62**	0.0023
ODF3B	Outer dense fiber of sperm tails 3B	34.6	261.0	106.0	773.8	**7.98**	$<1.0 \times 10^{-4}$	**3.28**	$<1.0 \times 10^{-4}$	**21.57**	$<1.0 \times 10^{-4}$
SLC15A3	Solute carrier family 15 member 3	16.3	119.4	54.7	607.4	**7.63**	$<1.0 \times 10^{-4}$	**3.45**	$<1.0 \times 10^{-4}$	**35.59**	$<1.0 \times 10^{-4}$
HAL	Histidine ammonia-lyase	6.2	44.1	28.7	47.4	**7.57**	$<1.0 \times 10^{-4}$	**4.71**	$<1.0 \times 10^{-4}$	**6.97**	$<1.0 \times 10^{-4}$
DOCK4	Dedicator of cytokinesis 4	44.0	306.8	144.9	139.2	**6.94**	$<1.0 \times 10^{-4}$	**3.21**	$<1.0 \times 10^{-4}$	**2.91**	$<1.0 \times 10^{-4}$
RAB27B	RAB27B, member RAS oncogene family	16.5	77.2	60.5	84.5	**5.98**	$<1.0 \times 10^{-4}$	**3.85**	$<1.0 \times 10^{-4}$	**5.62**	$<1.0 \times 10^{-4}$
CH25H	Cholesterol 25-hydroxylase	7.4	36.5	25.8	41.8	**4.41**	$<1.0 \times 10^{-4}$	**3.27**	0.022	**6.32**	$<1.0 \times 10^{-4}$
USP43	Ubiquitin specific peptidase 43	4.4	12.8	13.6	16.1	**3.94**	0.020	**3.41**	0.013	**4.50**	0.0091
AC104966.1	Ceruloplasmin (ferroxidase) (CP) pseudogene	16.5	47.6	57.3	53.7	**3.39**	$<1.0 \times 10^{-4}$	**3.79**	$<1.0 \times 10^{-4}$	**3.36**	$<1.0 \times 10^{-4}$
KLK10	Kallikrein related peptidase 10	14.0	37.1	33.0	43.1	**3.11**	0.022	**3.29**	0.0067	**2.65**	0.0028

Red = upregulated genes.

Table 6. Genes downregulated by all studied proinflammatory cytokines with FC < −2.5. Shown are mean normalized expression levels in control (Co), in C(IL1), C(IL17) and C(IFNγ) phenotypes, fold changes (FCs) for all comparisons vs. control and false discovery rate (FDR)-adjusted *p* values for them.

Gene	Name	Function	Mean exp. (Co)	Mean exp. (IL1)	Mean exp. (IL17)	Mean exp. (IFNγ)	FC (IL1 vs. Co)	adj. p (IL1 vs. Co)	FC (IL17 vs. Co)	adj. p (IL17 vs. Co)	FC (IFNγ vs. Co)	adj. p (IFNγ vs. Co)
SCN2B	Sodium voltage-gated channel beta subunit 2	Sodium ion transport	170.8	8.7	65.9	63.7	**−22.19**	$<1.0 \times 10^{-4}$	**−2.59**	$<1.0 \times 10^{-4}$	**−2.90**	$<1.0 \times 10^{-4}$
TNNT3	Troponin T3, fast skeletal type	Skeletal muscle constituent	97.8	6.4	17.9	31.6	**−17.51**	$<1.0 \times 10^{-4}$	**−5.28**	$<1.0 \times 10^{-4}$	**−3.26**	$<1.0 \times 10^{-4}$
MRAP2	Melanocortin 2 receptor accessory protein 2	Metabolism?	1348.7	91.1	376.5	572.0	**−15.12**	$<1.0 \times 10^{-4}$	**−3.62**	$<1.0 \times 10^{-4}$	**−2.85**	$<1.0 \times 10^{-4}$
WFDC1	WAP four-disulfide core domain 1	Negative regulation of cell growth	60.1	6.1	34.9	18.0	**−12.06**	$<1.0 \times 10^{-4}$	**−2.68**	0.0019	**−4.06**	$<1.0 \times 10^{-4}$
RANBP3L	RAN binding protein 3 like	Nuclear export	654.8	74.6	284.8	280.0	**−9.40**	$<1.0 \times 10^{-4}$	**−2.54**	$<1.0 \times 10^{-4}$	**−2.60**	$<1.0 \times 10^{-4}$
ASPN	Asporin	Skeletal system development, negative regulation of TGFβ signaling	2094.0	206.3	505.2	837.5	**−8.28**	$<1.0 \times 10^{-4}$	**−3.92**	$<1.0 \times 10^{-4}$	**−2.77**	$<1.0 \times 10^{-4}$
FGFR4	Fibroblast growth factor receptor 4	Cell proliferation and migration	10.3	2.3	3.1	5.1	**−5.59**	5.2×10^{-4}	**−3.12**	0.036	**−3.31**	0.045
PTGER3	Prostaglandin E receptor 3	Inflammation, cell death	494.1	173.6	162.3	188.8	**−2.69**	$<1.0 \times 10^{-4}$	**−3.03**	$<1.0 \times 10^{-4}$	**−2.82**	$<1.0 \times 10^{-4}$

blue = downregulated genes.

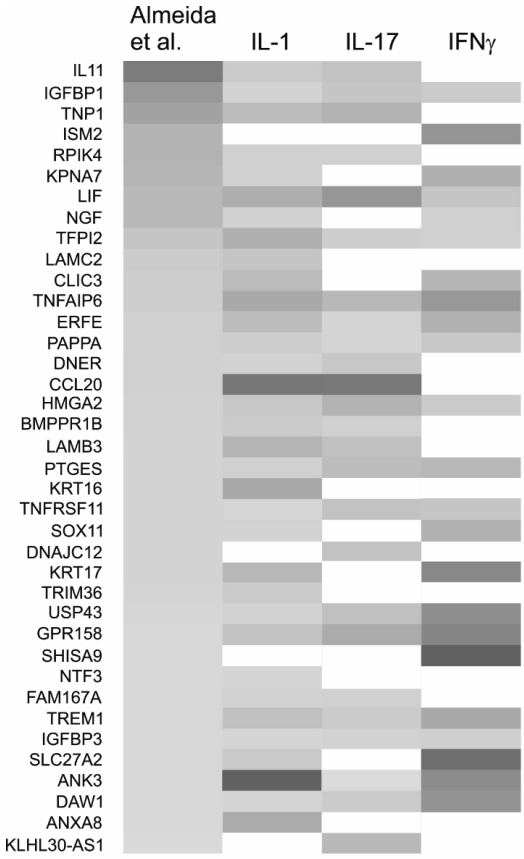

Figure 2. Heatmap of genes markedly upregulated (FC > 2.5) in degraded cartilage in the study by Almeida et al. [35] and significantly affected by at least one studied proinflammatory cytokine. Upregulated genes are marked with red, downregulated with blue, and genes with no significant fold change with white.

In the study by Almeida et al. [35], 52 genes were associated with preserved rather than degraded cartilage (i.e., significantly downregulated in degraded cartilage with FC < −2.5). Of these, 19 were significantly affected by at least one of the proinflammatory cytokines in our data. In C(IL-1β) cells, 13 of these 19 genes were significantly downregulated with *GDF10* displaying especially strong downregulation. In contrast, five of these genes were upregulated compared to control (including the especially strongly upregulated *C3* and *RSPO3*). This again shows that the net effect of IL-1β is to shift chondrocyte phenotype towards degraded cartilage. IFNγ showed a directionally similar, but less pronounced effect: seven of the genes associated with preserved cartilage were significantly downregulated and three upregulated in the C(IFNγ) phenotype. In C(IL-17) cells, eight genes associated with preserved cartilage were down- and four upregulated; *C3* once again displayed especially strong upregulation. Five genes, including *PTGER3* and *GDF10*, were downregulated in all of the three chondrocyte phenotypes. On the other hand, *RSPO3* and *PRLR*, both downregulated in degraded compared with preserved cartilage, were upregulated by all of the three cytokines. These data indicate that the C(IL-1β) and C(IL-17) phenotypes at least partly resemble the transcriptomic profile associated with degraded OA cartilage as identified by Almeida et al. [35]. In contrast, IFNγ seems to have a smaller effect

on the genes directly linked to cartilage degradation in OA being instead characterized by the upregulation of genes associated with antigen processing and presentation. (Figure 3 and Table S5).

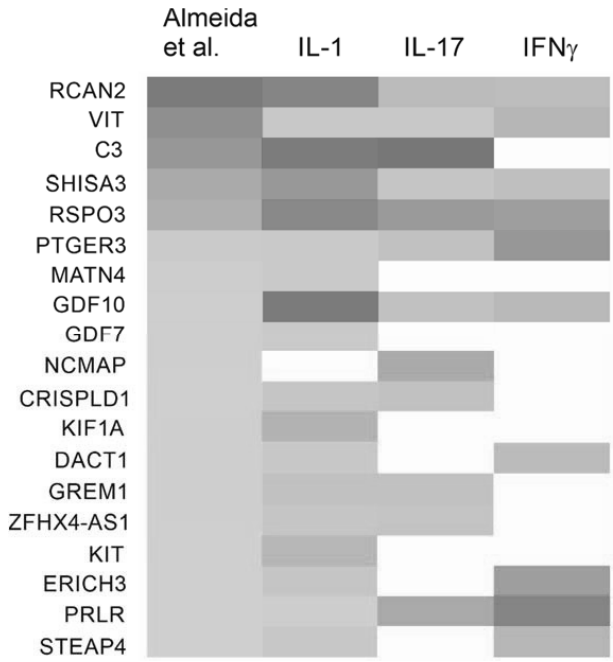

Figure 3. Heatmap of genes markedly downregulated (FC < −2.5) in degraded cartilage in the study by Almeida et al. [35] and significantly affected by at least one studied proinflammatory cytokine. Upregulated genes are marked with red, downregulated with blue, and genes with no significant fold change with white.

Relatively few genes were significantly affected by IL-4 in our data, and none of them were markedly (with FC > 2.5) associated with either degraded or preserved cartilage in the data of Almeida et al. [35]. However, looking at genes with a smaller proportional difference between degraded and preserved cartilage (FC > 1.5 in either direction) produced several genes that were significantly affected by IL-4. Ten genes (including *DUSP5* and *COL7A1*) were upregulated in degraded cartilage and also upregulated in C(IL-4) cells. In contrast, one gene associated with degraded cartilage (*HMMR*) was downregulated by IL-4, and seven genes (including *COL14A1*) associated with preserved cartilage were upregulated by IL-4. (Table S6)

To demonstrate that naïve chondrocytes can be affected by the cytokines studied, we separately studied the expression of their receptors. As shown in Table S7, receptors for all studied cytokines were expressed in unstimulated OA chondrocytes at meaningful levels.

3. Discussion

Chondrocytes from OA patients were found to adopt distinct phenotypes in response to the central $T_H1/T_H2/T_H17$ cytokines. The phenotype induced by the T_H1 cytokine interleukin 1 (IL-1β), the C(IL-1β) phenotype, can be characterized by widespread, strong upregulation of inflammation and catabolism as well as downregulation of metabolic signaling. The effects of the T_H17 cytokine IL-17 appear to be somewhat less widespread and partly overlapping those of IL-1β, with induction of inflammatory and chemotactic factors. The phenotype induced by the second T_H1 cytokine interferon gamma (IFNγ)

seems to be distinct from both C(IL-1β) and C(IL-17) phenotypes, with a significant theme of antigen processing and presentation. The effects of the T_H2 cytokine IL-4 were much more modest; some factors involved in the regulation of inflammation and TGFβ signaling were upregulated, while the downregulated genes were mostly associated with cell proliferation and migration.

In T cells, the T_H1 phenotype drives inflammation and defense against intracellular pathogens (cell-mediated immunity) and is associated with the production of proinflammatory cytokines such as IFNγ and IL-1β [36]. Conversely, T_H2 cells promote humoral immunity, regulate inflammation and direct resolving and injury-healing responses [11]. Central T_H2 cytokines are IL-4 and IL-13. A third relatively well-established population of T_H cells is the T_H17 phenotype. These cells produce IL-17, drive autoimmune reactions and activate neutrophils. This contrasts with T_H1 cells that preferentially affect monocytes/macrophages, as well as T_H2 cells that are associated with eosinophils, basophils and mast cells [37].

The central $T_H1/T_H2/T_H17$ cytokines also induce loosely analogous macrophage phenotypes. Like T_H1 cells, M1 or "classically activated" macrophages are induced by proinflammatory cytokines such as IL-1β and IFNγ and promote inflammation by secreting further proinflammatory factors. M2 or "alternatively activated" macrophages are induced canonically by IL-4. In addition to functioning as antiparasite effectors, they attenuate inflammation, direct wound-healing processes and promote the resolution of inflammation. [38] IL-17 induces a less-studied macrophage phenotype characterized by increased chemotaxis and the production of proinflammatory factors such as cyclooxygenase 2 (COX-2), IL-6 and tumor necrosis factor alpha (TNFα) [15,39] as well as resolution-promoting effects in the later phases of inflammation [16].

The chondrocyte phenotypes induced by different cytokines in our study can be considered analogous to T_H cell and particularly macrophage phenotypes. IL-1β affects a very large number of genes and induces a phenotype characterized by the expression of inflammatory and matrix-degrading genes. The C(IL-17) phenotype appears likewise proinflammatory, but with a somewhat more limited repertoire of inflammatory genes. C(IFNγ) also appears to be a phenotype that is inflammatory, but is also characterized by genes linked to antigen presentation. The C(IL-4) phenotype is characterized by the expression of genes linked to TGFβ signaling and the regulation of inflammation.

The chondrocyte phenotypes induced by the $T_H1/T_H2/T_H17$ cytokines appeared to be quite distinct as only 45 genes were markedly (FC > 2.5) upregulated and eight markedly downregulated (FC < −2.5) by all three proinflammatory cytokines, considering that hundreds of genes were up- and dozens downregulated to a similar extent by each of the three cytokines. The factors upregulated by all of the three proinflammatory cytokines (IL-1β, IFNy and IL-17) include the well-known inflammatory mediators *IL6*, nitric oxide synthase 2/inducible nitric oxide synthase (*NOS2*/iNOS) and prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 (*PTGS2*/COX-2). On this list were also included, for example, pentraxin 3 (*PTX3*), toll-like receptor 2 (*TLR2*), chemokine (C-C motif) ligand 2 (*CCL2*), interferon regulatory factor 4 (*IRF4*) and prolactin receptor (*PRLR*). Pentraxin 3 (*PTX*) promotes inflammation by activating the classical complement pathway and by facilitating antigen recognition by mononuclear phagocytes [40], and it has been shown to be elevated in the serum and synovial fluid of patients with rheumatoid arthritis [41]. *TLR2* is a pattern recognition receptor mediating innate immune activation by microbial particles. In osteoarthritis, it is activated by hyaluronan and aggrecan fragments leading to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, which may contribute to OA progression and pain [42,43]. *CCL2* is a monocyte-attracting chemokine that has been linked to OA development and pain [44,45]. *IRF4* has recently been associated with cartilage destruction and pain in OA via the induction of CCL17 [46]. Prolactin has been implicated to promote chondrocyte differentiation and attenuate apoptosis, and thus the upregulation of its receptor might promote cartilage survival [47,48].

Factors downregulated by all of the three proinflammatory cytokines include asporin (*ASPN*) and prostaglandin EP3 receptor (*PTGER3*). Asporin belongs to the family of leucine-rich repeat proteins and is associated with cartilage matrix, also bearing a similarity to decorin [49]. The potential role of asporin in OA appears to be unclear; several studies have linked the protein to the development of the disease, where it might impair chondrogenesis by inhibiting TGF-β signaling [50]. Polymorphisms of the asporin gene have also been linked to OA risk [51], even though the most recent meta-analysis failed to find evidence for this [52]. Prostaglandin E2 (PGE2)-induced *PTGER3* downregulation may contribute to cartilage inflammation and damage via NF-κB activation and IL-6 synthesis [53].

When the Gene Ontology (GO) terms significantly affected by the three different proinflammatory cytokines were studied, all three were found to affect those associated with inflammation. IL-1β was alone in significantly affecting several terms, such as cell adhesion, extracellular matrix metabolism and collagen catabolism, linking the chondrocyte phenotype induced by this cytokine to these functions. IL-17 solely affected nitric oxide synthase biosynthesis. This is intriguing, as the nitric oxide production is an important part of inflammatory response in chondrocytes [54]. The C(IFNγ) phenotype seems to be differentiated from others by activation of pathways related to antigen processing and presentation. Chondrocytes are not considered "professional" antigen-presenting cells, but they have, interestingly, been shown to present cartilage proteoglycans as antigens to CD8+ T cells, potentially contributing to local joint inflammation [55,56].

Previously published genome-wide expression analyses (GWEAs) have identified a number of differentially expressed genes between either damaged and intact OA cartilage or healthy and OA cartilage. These include genes involved in inflammation, skeletal system development, cell adhesion and monosaccharide metabolism [35,57–59]. When comparing our results to those of the comprehensive study by Almeida et al. [35], the C(IL-1β) phenotype most closely resembled degraded OA cartilage, while IL-17 upregulated a smaller number of proinflammatory factors associated with degraded cartilage in that study. Accordingly, some genes associated with preserved as opposed to degraded cartilage were also downregulated by these proinflammatory cytokines. Most of these genes are linked to cartilage anabolism. The effects of IFNγ and (especially) IL-4 on the genes identified by Almeida et al. [35] were more modest. It is important to note that the receptors for all cytokines studied were expressed at marked levels in our samples, which lends further validity to our results.

A potential limitation of the study is that whole thickness pieces of cartilage obtained from joint replacement surgery were used for chondrocyte isolation. Thus, the cells obtained are likely a mixture of chondrocytes from different layers of cartilage, and there might be some differences in the effects of cytokines between these groups. However, all chondrocytes can be expected to be exposed to cytokines diffused from the synovial fluid and/or produced by chondrocytes (in autocrine or paracrine manner). Thus, we think that the observed clear differences in the chondrocyte phenotypes in response to the major $T_H1/T_H2/T_H17$ cytokines are relevant for further understanding of chondrocyte biology and OA pathophysiology. In future studies, cartilage layer-specific cell isolation methods or single-cell RNA-Seq could be considered to unravel possible zone-specific responses.

Another limitation of the study is that the chondrocytes used were obtained from OA joints; therefore, some of the detected effects of the cytokines might differ from those observed in healthy chondrocytes. Studying the effects of the cytokines on healthy chondrocytes would be an interesting avenue of future study; however, obtaining healthy primary human chondrocytes presents a practical challenge (compared to OA chondrocytes which can be obtained from joint replacement surgery). In the present study, we observed similarities between the C(IL-1β) and C(Il-17) phenotypes and the gene expression profile of chondrocytes from degraded OA cartilage published by Almeida et al. [35]; C(IFNγ) and especially C(IL-4) bore less resemblance to that phenotype. This suggests that the cytokine-induced phenotypes observed in our data have relevance regarding OA pathogenesis.

In conclusion, OA chondrocytes, analogously to macrophages, can assume distinct phenotypes in response to the cytokines associated with the $T_H1/T_H2/T_H17$ phenotypes of T helper cells. These results provide novel information on chondrocyte biology and the pathogenesis of OA with further insights into the development of disease-modifying drugs for (osteo)arthritis.

4. Materials and Methods

4.1. Cartilage and Cell Culture

Leftover cartilage pieces were collected from nine patients undergoing total knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. All patients fulfilled the American College of Rheumatology classification criteria for knee OA [60]. Patients with diabetes mellitus were excluded from the study to avoid potential confounding effects on chondrocyte metabolism [61]. The study was approved by the Ethics Committee of Tampere University Hospital, Finland, and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients. Chondrocyte isolation and culture was carried out as previously described [62]. To ensure an adequate yield of chondrocytes, all available cartilage was removed aseptically using a scalpel from the bony parts received from joint replacement surgery and cut into small pieces. The pieces were first washed with phosphate buffered saline (PBS). After that, they were incubated for 24 h in the presence of Liberase enzyme (Roche, Mannheim, Germany) 0.25 mg/mL, diluted in serumless Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) with glutamax-I containing penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (all three from Invitrogen, Carlsbad, CA, USA) at 37 °C. The resulting cell suspension was poured through a 70 μm nylon mesh and centrifuged for five minutes at 200 g. Cells were then washed twice and seeded on 24-well plates (0.2 million cells/mL) in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Lonza) together with the aforementioned compounds. Confluent cultures were exposed to fresh culture medium alone, with 10 ng/mL IFNγ, with 100 pg/mL IL-1β, with 50 ng/mL IL-17 or with 10 ng/mL IL-4, for 24 h. The concentrations used were chosen based on our preliminary experiments with cultured chondrocytes.

4.2. RNA Isolation and Sample Preparation

Culture medium was removed at the indicated time points and total RNA of the chondrocytes was extracted with GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). The sample was treated with DNAse I (Fermentas UAB, Vilnius, Lithuania). RNA concentration and integrity were confirmed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

4.3. Next Generation Sequencing and Data Analysis

Sequencing of samples was performed in the Finnish Institute of Molecular Medicine (FIMM) sequencing core, Helsinki, Finland, using the Illumina HiSeq 2500 sequencing platform. Sequencing depth was 20 million paired-end reads 100 bp in length. Read quality was first assessed using FastQC [63], and the reads were trimmed using Trimmomatic [64]. Trimmed reads were aligned to reference human genome with STAR [65]. Count matrices were prepared with the featureCounts program [66]. Differential expression was assessed with DESeq2 [67]. Gene expression levels were given as DeSeq2-normalized counts, and genes with an average normalized count 10 or less across all samples were excluded from further analysis. For the purposes of further analysis, genes with a minimum of 2.5 fold change (FC) in abundance and FDR-corrected p-value < 0.05 were deemed biologically and statistically significant (unless otherwise indicated). Functional analysis was performed against the Gene Ontology (GO) database [68,69] using the DAVID tool [70], and REVIGO was used to reduce the resulting list [71].

4.4. Statistics

For NGS data analysis, normalization was performed and differential expression studied using a negative binomial model implemented in DESeq2.

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Data Availability Statement: Complete gene expression data for all samples are available from the corresponding author upon reasonable request.

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PUBLICATION

IV

Widespread regulation of gene expression by glucocorticoids in chondrocytes from patients with osteoarthritis as determined by RNA-Seq

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Widespread regulation of gene expression by glucocorticoids in chondrocytes from patients with osteoarthritis as determined by RNA-Seq

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Abstract

Background: Intra-articular glucocorticoid (GC) injections are widely used as a symptomatic treatment for osteoarthritis (OA). However, there are also concerns about their potentially harmful effects, and their detailed effects on chondrocyte phenotype remain poorly understood.

Methods: We studied the effects of dexamethasone on gene expression in OA chondrocytes with RNA-Seq. Chondrocytes were isolated from the cartilage from OA patients undergoing knee replacement surgery and cultured with or without dexamethasone for 24 h. Total RNA was isolated and sequenced, and functional analysis was performed against the Gene Ontology (GO) database. Results for selected genes were confirmed with RT-PCR. We also investigated genes linked to OA in recent genome-wide expression analysis (GWEA) studies.

Results: Dexamethasone increased the expression of 480 and reduced that of 755 genes with a fold change (FC) 2.0 or greater. Several genes associated with inflammation and cartilage anabolism/catabolism as well as lipid and carbohydrate metabolism were among the most strongly affected genes. In the GO analysis, genes involved in the extracellular matrix organization, cell proliferation and adhesion, inflammation, and collagen synthesis were enriched among the significantly affected genes. In network analysis, NGF, PI3KR1, and VCAM1 were identified as central genes among those most strongly affected by dexamethasone.

Conclusions: This is the first study investigating the genome-wide effects of GCs on the gene expression in OA chondrocytes. In addition to clear anti-inflammatory and anticatabolic effects, GCs affect lipid and glucose metabolism in chondrocytes, an observation that might be particularly important in the metabolic phenotype of OA.

Keywords: Osteoarthritis, Cartilage, Chondrocyte, Glucocorticoid, RNA-Seq

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Introduction

Osteoarthritis (OA) is a disease that affects over 15% of the global population aged 60 or more, causing pain, disability, and reduced quality of life, as well as major costs to healthcare systems [1]. The disease process in the joint is characterized by oxidative stress, low-grade inflammation, and increased catabolism. This eventually results in the breakdown of the articular cartilage and changes in other tissues of the joint, leading to pain and loss of function [2].

Chondrocyte gene expression is markedly altered in osteoarthritis [3]. Some of these changes are thought to be harmful (such as increased expression of proteolytic enzymes and proinflammatory cytokines) and some protective (e.g., increased expression of extracellular matrix [ECM] components) [4]. A couple of genome-wide expression analyses (GWEAs) have previously been performed comparing damaged OA cartilage either to intact OA cartilage or to cartilage from a healthy donor [5–8]. Two larger studies have utilized microarrays and RNA-Seq respectively to compare lesioned and healthy OA cartilage in the same joint, identifying a number of differentially expressed genes involved in inflammation, skeletal system development, cell adhesion, and monosaccharide metabolism [9, 10].

As no proven disease-modifying medications are currently available for OA, all pharmacological treatments are essentially symptomatic [1]. Intra-articular injections with glucocorticoids (GCs) are widely used to combat inflammation and pain and are recommended in the OARSI [11], ACR [12], and NICE [13] treatment guidelines for the management of knee OA. However, their long-term benefits are unclear, and there appears to be a significant variation in the responses between individual patients [14]. GCs are thought to suppress harmful low-grade inflammation present in OA joints. However, there are some concerns about potentially deleterious long-term effects [15, 16].

Glucocorticoids are steroid hormones that are endogenously produced in the adrenal cortex. In target cells, they form a complex with glucocorticoid receptor (GR), which then dimerizes and migrates into the nucleus. After that, GCs cause their anti-inflammatory effects through two main mechanisms. The GR-steroid complex binds to glucocorticoid response elements (GREs) in the promoter region of target genes, promoting the expression of anti-inflammatory genes as well as a number of genes involved in various other functions. In addition, GCs can inhibit the activity of inflammatory transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) and sequester their coactivators, leading to suppression of the expression of various inflammatory genes and attenuation of inflammation [17]. In chondrocytes, glucocorticoids have also been shown to reduce chondrocyte viability by inducing oxidative stress and apoptosis [18]. In addition to their effects on inflammation, modulation of glucose and lipid metabolism by glucocorticoids could mediate some of their effects on OA cartilage. Osteoarthritis is linked to metabolic disturbances such as obesity and diabetes, giving rise to the concept of a so-called metabolic phenotype of OA. There is also evidence on metabolic derangements in articular chondrocytes, and impairments in, for example, glycolysis [19], cholesterol metabolism, [20], and mitochondrial respiration [21] have been reported. Due to their established effects on these metabolic pathways in other cell types [22], glucocorticoids could plausibly affect OA pathogenesis via affecting chondrocyte metabolism.

In the present study, we set out to study the effects of the glucocorticoid dexamethasone on gene expression in OA chondrocytes. The aim was to identify significantly modulated pathways and/or functional categories of genes that might be important in the pathogenesis of OA. The results were also compared to those of two previous GWEA studies based on the patient material of the RAAK study [9, 10] to determine whether glucocorticoid treatment might "shift" the expression profile of OA chondrocytes towards the one in healthy cells. In addition, we studied the effects of dexamethasone on potential OA susceptibility genes previously identified in genome-wide association (GWAS) studies [23–25].

Methods

Patients and cell culture

Leftover cartilage pieces were collected from OA patients (n = 10, BMI 27.3 [5.8] kg/m^2, age 70.0 [14.8] years, median [IQR]; 4/6 females/males) undergoing total knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. All patients fulfilled the American College of Rheumatology classification criteria for knee OA [26] with a mean Kellgren-Lawrence score of 3.5 (SEM 0.22). Patients with diabetes mellitus were excluded from the study.

Chondrocyte isolation and culture were performed as previously described [27]. The articular cartilage was removed aseptically from the subchondral bone using a scalpel and cut into small pieces. The pieces were first washed with phosphate-buffered saline (PBS). After that, they were incubated overnight in the presence of Liberase™ enzyme (Roche Applied Science, Penzberg, Germany, 0.25 mg/mL) diluted in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) with GlutaMAX-I containing penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (all three from Invitrogen, Carlsbad, CA, USA) at 37 °C. The resulting cell suspension was poured through a 70-μm nylon mesh and centrifuged for 5 min at 1500 rpm.

The cells were washed and seeded on 24-well plates (0.2 million cells/mL) and incubated for 24 h. Thereafter, the experiments were started by adding dexamethasone (1 μM) in the fresh culture medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum [Lonza] together with the aforementioned substituents) for 24 h.

Enzyme-linked immunosorbent assay

A separate group of nine cartilage samples was used for ELISA measurements. Cartilage preparation, chondrocyte isolation, and incubation were performed as described above. After 24 h, incubations were terminated by collecting the cell culture media, and culture medium samples were stored at − 20 °C until analyzed. The concentrations of human MMP-1, MMP-13, and CCL2 (MCP-1) were determined by enzyme-linked immunosorbent assay (ELISA) by using reagents from R&D Systems Europe Ltd., 175 Abingdon, UK (catalog nos. DY901, DY511, and DY279, respectively). The detection limits were 39 pg/mL, 31.3 pg/mL, and 7.8 pg/mL, respectively.

RNA isolation and sample preparation

The culture medium was removed at the indicated time point, and total RNA of the chondrocytes was extracted with GenElute™ Mammalian Total RNA Miniprep kit (Sigma). Total RNA was treated with DNAse I (Qiagen, Hilden, Germany). RNA concentration and integrity were confirmed with the 2100 Bioanalyzer (Agilent Technologies). RNA (150 ng/sample) was reverse-transcribed to cDNA for RT-PCR using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA).

Next-generation sequencing and data analysis

Sequencing of RNA samples (500 ng) was performed in the Finnish Institute of Molecular Medicine (FIMM) sequencing core, Helsinki, Finland, using the Illumina HiSeq 2500 sequencing platform. Sequencing depth was 15 million paired-end reads 100 bp in length. Read quality was first assessed using FastQC [28], and the reads were trimmed using Trimmomatic [29]. Trimmed reads were aligned to the full reference human genome with STAR [30]. Count matrices were prepared with the featureCounts program [31]. Differential expression was assessed with DESeq2 using patient number as an additional experimental factor for pairwise comparisons [32]. Gene expression levels are given as DESeq2-normalized counts, and genes with a mean normalized count 5 or less across all samples were excluded from further analysis. For the purposes of further analysis, genes with a minimum of 2.0 fold change (FC) in abundance and false discovery rate (FDR)-corrected p value < 0.05 were deemed biologically and statistically significant. Functional analysis was performed against the Gene Ontology (GO) database [33] using ranked list enrichment implemented in the GOrilla tool [34], and protein interactions were studied with STRING [35]. Gene functions were obtained from the NCBI Gene database.

Quantitative reverse transcription/polymerase chain reaction

cDNA obtained from the reverse transcriptase reaction was diluted 1:20 with RNAse-free water and subjected to quantitative RT-PCR using TaqMan Universal PCR Master Mix and the ABI Prism 7500 Sequence detection system (Applied Biosystems).

Primers and probes for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclooxygenase-2 (COX-2), MAP kinase phosphatase 1 (MKP-1), matrix metalloproteinases (MMPs) 1 and 13, collagen type II, alpha 1 (COL2A1), and aggrecan (ACAN) were obtained from Metabion International AG (Martinsried, Germany). The primer and probe sequences (Table S1) and concentrations were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. mRNA levels of other studied genes were determined with TagMan Gene Expression Assays (Applied Biosystems): MMP16 (assay number Hs00234676_m1), collagen type IX, alpha 1 (COL9A1, Hs00932136_g), collagen type XI, alpha 1 (COL11A1, Hs01097664_m1), C-C motif chemokine ligand 2 (CCL2, Hs00234140_m1), MAP kinase phosphatase 2 (MKP-2, Hs01027785_m1), Kruppel like factor 9 (KLF9, Hs00230918_m1), nerve growth factor (NGF, Hs00171458_m1), tumor necrosis factor superfamily member 15 (TNFSF15, Hs00270802_s1), and forkhead box O3 (FOXO3, Hs00818121_m1).

PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each experimental reaction was performed in duplicate. The relative mRNA levels of genes listed in Table S1 were quantified using the standard curve method as described in Applied Biosystems User Bulletin number 2. To calculate the relative expression of mRNAs determined with TaqMan assays, the $2^{(-\Delta\Delta CT)}$ method was used. According to the method, the cycle threshold (C_T) value for genes of each gene was normalized to the C_T value of GAPDH mRNA in the same sample.

Statistics

For NGS data analysis, normalization was performed and differential expression studied using a negative binomial model implemented in DESeq2. In ELISA and PCR

Table 1 Twelve most strongly up- and downregulated genes in dexamethasone-treated OA chondrocytes (D) relative to control (Co)

Genes most strongly upregulated by dexamethasone						
Gene	**Name**	**Function**	**Mean (Co)**	**Mean (D)**	**Fold change**	**FDR*p***
GPM6B	Glycoprotein M6B	Regulation of osteoblast function	205.4	8033.4	**37.53**	< 0.0001
SULT1B1	Sulfotransferase family 1B member 1	Sulfation	14.5	702.2	**34.30**	< 0.0001
KLRD1	Killer cell lectin like receptor D1	Regulation of inflammation	15.1	685.0	**29.24**	< 0.0001
FLRT3	Fibronectin leucine rich transmembrane protein 3	Cell adhesion	6.9	247.4	**27.28**	< 0.0001
FAM83A-AS1	FAM83A antisense RNA 1	Regulation of cell proliferation	0.4	43.6	**21.41**	< 0.0001
ZBTB16	Zinc finger and BTB domain containing 16	Cartilage development	113.9	2490.2	**21.11**	< 0.0001
FKBP5	FK506 binding protein 5	Glucocorticoid receptor regulation	702.8	15089.3	**20.11**	< 0.0001
PDK4	Pyruvate dehydrogenase kinase 4	Glucose and lipid metabolism	251.6	5804.9	**19.84**	< 0.0001
PTK2B	Protein tyrosine kinase 2 beta	Regulation of cell proliferation	116.6	2393.8	**19.43**	< 0.0001
ABCD2	ATP binding cassette subfamily D member 2	Lipid metabolism	13.8	340.3	**18.77**	< 0.0001
HAL	Histidine ammonia-lyase	Histidine metabolism	5.5	176.0	**16.34**	< 0.0001
GLUL	Glutamate-ammonia ligase	Cell proliferation	3886.9	64025.9	**15.67**	< 0.0001
Genes most strongly downregulated by dexamethasone						
Gene	**Name**	**Function**	**Mean (Co)**	**Mean (D)**	**Fold change**	**FDR*p***
PLAU	Plasminogen activator, urokinase	Cell proliferation and migration	169.7	9.5	**-14.03**	< 0.0001
INHBA	Inhibin beta A subunit	Cell proliferation and differentiation	26339.5	2220.8	**-11.71**	< 0.0001
P2RY6	Pyrimidinergic receptor P2Y6	Inflammation	114.3	7.6	**-10.56**	< 0.0001
VCAM1	Vascular cell adhesion molecule 1	Inflammation	24663.5	2854.2	**-8.51**	< 0.0001
GREM1	Gremlin 1, DAN family BMP antagonist	Chondrocyte proliferation and differentiation	6327.8	691.8	**-8.22**	< 0.0001
HAS2	Hyaluronan synthase 2	ECM production	1922.7	234.2	**-8.11**	< 0.0001
SLC26A4	Solute carrier family 26 member 4	Anion transport	1537.7	180.9	**-7.73**	< 0.0001
ARSI	Arylsulfatase family member I	Glycosphingolipid metabolism	102.6	11.7	**-7.46**	< 0.0001
MXRA5	Matrix remodeling associated 5	TGFβ signaling	1100.1	132.2	**-7.31**	< 0.0001
MYBPH	Myosin binding protein H	Cell adhesion	57.6	5.7	**-7.21**	< 0.0001
TSPAN2	Tetraspanin 2	Cell migration	1192.6	154.4	**-7.11**	< 0.0001
NGF	Nerve growth factor	Pain	129.1	16.2	**-6.92**	< 0.0001

FDR*p* = False discovery rate -corrected *p*-value

experiments, paired Student's *t* test was used to assess the statistical significance of differential expression, and multiple testing was addressed using Bonferroni correction. Data is presented as average + standard error of the mean (SEM).

Results

Differentially expressed genes

After normalization and correction for multiple testing, 480 genes were upregulated more than 2.0-fold in dexamethasone-treated cells compared to control cells, and 755 downregulated by the same factor (FC < − 2.0). In total, 7371 genes were found to be differentially expressed in dexamethasone-treated versus control cartilage in a statistically significant manner (FDR-corrected p value < 0.05). Of these, 3612 were up- and 3759 downregulated. Twelve most strongly up- and downregulated genes are listed in Table 1. A complete list of differentially expressed genes in dexamethasone-treated chondrocytes compared to untreated cells is provided in the Supplementary data in Table S2.

The list of the most strongly upregulated genes includes genes involved in the regulation of cell proliferation, inflammation, cartilage development, and carbohydrate and lipid metabolism. Among the most strongly downregulated genes are those linked to cell proliferation and differentiation, extracellular matrix production, and inflammation. Also included was nerve growth factor (NGF), a known mediator of OA pain [36] (Table 1).

Table 2 Ranked Gene Ontology (GO) terms significantly affected by dexamethasone

GO term	Description	FDR *p*-value
GO:0048523	Negative regulation of cellular process	3.50E-08
GO:0032502	Developmental process	3.81E-08
GO:0048519	Negative regulation of biological process	4.05E-08
GO:0030334	Regulation of cell migration	5.17E-08
GO:0051239	Regulation of multicellular organismal process	1.69E-07
GO:0032501	Multicellular organismal process	4.37E-07
GO:0007166	Cell surface receptor signaling pathway	4.66E-07
GO:0043062	Extracellular structure organization	6.75E-06
GO:0030198	Extracellular matrix organization	7.34E-06
GO:0048856	Anatomical structure development	7.86E-06
GO:0032879	Regulation of localization	7.91E-06
GO:0009725	Response to hormone	1.25E-05
GO:0042127	Regulation of cell proliferation	1.27E-05
GO:0050896	Response to stimulus	3.12E-05
GO:0040011	Locomotion	0.000137
GO:0048518	Positive regulation of biological process	0.000193
GO:0050794	Regulation of cellular process	0.000197
GO:0040008	Regulation of growth	0.000199
GO:0048583	Regulation of response to stimulus	0.000351
GO:0065008	Regulation of biological quality	0.000584
GO:0043408	Regulation of MAPK cascade	0.00105
GO:0009719	Response to endogenous stimulus	0.00152
GO:0006954	Inflammatory response	0.00235
GO:0006950	Response to stress	0.00241
GO:0065007	Biological regulation	0.00281
GO:0051174	Regulation of phosphorus metabolic process	0.00303
GO:0010273	Detoxification of copper ion	0.00306
GO:0048878	Chemical homeostasis	0.0032
GO:0010941	Regulation of cell death	0.00426
GO:0070482	Response to oxygen levels	0.00506
GO:0061687	Detoxification of inorganic compound	0.0057
GO:0006928	Movement of cell or subcellular component	0.00578
GO:0022610	Biological adhesion	0.00626
GO:0009611	Response to wounding	0.0064
GO:0007155	Cell adhesion	0.00717
GO:0065009	Regulation of molecular function	0.0116
GO:0051338	Regulation of transferase activity	0.023
GO:0006068	Ethanol catabolic process	0.0239
GO:0019216	Regulation of lipid metabolic process	0.0255
GO:0009628	Response to abiotic stimulus	0.0342
GO:0006109	Regulation of carbohydrate metabolic process	0.0375
GO:0051246	Regulation of protein metabolic process	0.039
GO:0002682	Regulation of immune system process	0.0425
GO:0032964	Collagen biosynthetic process	0.0429
GO:0008283	Cell proliferation	0.0454

FDR *p*-value = False discovery rate -corrected *p*-value

Next, we studied, by ranked list enrichment GO analysis, which functional gene categories were enriched among those genes with the largest fold changes into either direction (Table 2). These include, among others, those associated with extracellular matrix organization, regulation of cell proliferation, inflammatory response, cell adhesion, MAP kinase signaling, collagen synthesis, and lipid and carbohydrate metabolism.

Genes involved in inflammation, oxidative stress, and extracellular matrix production

As low-grade inflammation, oxidative stress, and changes in extracellular matrix production and catabolism are central features in the pathogenesis of OA, we set out to separately study genes linked to these processes (Table 3). Several proinflammatory factors such as cyclooxygenase-2 (COX-2, fold change − 4.29), chemokine (C-C motif) ligand 2 (CCL2, fold change − 5.43), and TNF superfamily member 15 (TNFSF15, fold change − 5.98) were significantly downregulated by dexamethasone, while the anti-inflammatory MAP kinase phosphatases 1 (MKP-1, fold change 10.48) and 2 (MKP-2, fold change 5.50) were upregulated. Genes affecting response to oxidative stress, such as Kruppel-like factor 9 (KLF9, fold change 10.85) and forkhead box O3 (FOXO3, fold change 4.29), were similarly upregulated. Also, the catabolic matrix metalloproteinases 1, 16, and 13 (MMP1 with fold change − 2.85, MMP16 with fold change − 3.12 and MMP13 with fold change − 4.08) were downregulated by dexamethasone. Various collagens were downregulated by dexamethasone, including the most highly expressed collagens COL2A1 (fold change − 2.28) and COL11A1 (fold change − 3.12). However, aggrecan was found to be significantly upregulated (fold change 2.43). The expression of connective tissue growth factor (CTGF, fold change 2.25) was enhanced while fibroblast growth factor 1 (FGF1, fold change − 2.62), transforming growth factor beta 2 (TGFB2, fold change − 2.57), and vascular endothelial growth factor A (VEGFA, fold change − 2.36) were downregulated by dexamethasone. The change in the expression of selected genes was confirmed with RT-PCR (Table S3) and the production of MMPs 1 and 13 as well as CCL2 with ELISA (Figure S1).

Carbohydrate and lipid metabolism

As glucocorticoids regulate glucose and lipid metabolism, and OA is known to be associated with metabolic syndrome, we separately studied genes for proteins participating in the main pathways of carbohydrate and lipid metabolism (glycolysis, oxidative phosphorylation, lipolysis, and beta-oxidation) [37]. Dexamethasone did not have a marked (fold change > 2.0) effect on any of these genes, with the sole exception being upregulation of long-chain acyl-CoA dehydrogenase (ACADL) (fold change 2.60) (Table S4).

However, dexamethasone affected the expression of several other genes regulating lipid and carbohydrate metabolism (those associated with hierarchically high-level GO terms GO:0019216 Regulation of lipid metabolism and GO:0006109 Regulation of carbohydrate metabolism). For example, pyruvate dehydrogenase kinase 4 (PDK4) and glycogen phosphorylase L (PYGL), as well as the redox regulator Sestrin 3 (SESN3), were markedly upregulated by dexamethasone (Fig. 1).

Integration with previous GWAS and GWEA studies

When the 53 genes previously associated with OA in GWAS studies [23–25] were studied separately, 12 of them were found to be significantly affected by dexamethasone with a FC greater than 2.0 (Table S5). Eleven of them (including COL11A1, COX-2, GDF5, IL6, and VEGFA) were downregulated and only one, IL16, upregulated.

The microarray-based GWEA study by Ramos et al. [9] identified 18 genes that were differentially expressed between degraded and preserved OA cartilage in the same joint, and two of them were affected by dexamethasone in our data (Table S6). Of these, the expression of COL9A1 was found to be lower in degraded cartilage, and the gene was also downregulated by dexamethasone. Nerve growth factor (NGF), in turn, was expressed at higher levels in degraded cartilage and strongly downregulated by dexamethasone.

Almeida et al. [10] analyzed gene expression in degraded and spared OA cartilage with NGS. In their data, 372 genes were differentially expressed with FC > 2.0 into either direction, and 78 of them were significantly affected by dexamethasone (FC > 2.0 into either direction) in the present study. Of these, 19 were upregulated in degraded cartilage compared with spared cartilage, and upregulated by dexamethasone in our data, while 25 were upregulated in degraded cartilage and downregulated by dexamethasone. Seventeen were downregulated in degraded cartilage and by dexamethasone, while another 17 were downregulated in degraded cartilage and upregulated by dexamethasone. Interestingly, NGF was one of the genes whose expression was enhanced in degraded OA cartilage and normalized by dexamethasone (Table S7).

Interactions between the differentially expressed genes

Among the genes most strongly affected by dexamethasone (FC > 5.0), several interactions were identified using the STRING database (Fig. 2). Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3KR1), vascular cell adhesion protein 1 (VCAM1), KIT proto-oncogene receptor tyrosine kinase (KIT), FGR proto-oncogene Src family tyrosine kinase (FGR), C-C motif ligand 2 (CCL2), and nerve growth factor (NGF) were found to occupy central positions in the interaction network.

Table 3 Selected genes linked to inflammation, oxidative stress, catabolic enzymes, and extracellular matrix production as well as growth factors in dexamethasone-treated OA chondrocytes (D) relative to control (Co)

Inflammation and oxidative stress

Gene	Name	Mean (Co)	Mean (D)	Fold Change	FDR *p*
KLF9	Kruppel like factor 9	582.2	6410.9	**10.85**	< 0.0001
MKP-1	MAP kinase phosphatase 1	695.1	7460.0	**10.48**	< 0.0001
MKP-2	MAP kinase phosphatase 2	227.2	1198.1	**5.50**	< 0.0001
TLR2	Toll like receptor 2	120.3	617.0	**4.72**	< 0.0001
FOXO3	Forkhead box O3	1541.3	6657.3	**4.29**	< 0.0001
IRF4	Interferon regulatory factor 4	27.6	128.5	**3.97**	< 0.0001
IL1R1	Interleukin 1 receptor type 1	3690.2	14009.3	**3.81**	< 0.0001
PPARG	Peroxisome proliferator activated receptor gamma	24.5	79.5	**3.12**	< 0.0001
IL16	Interleukin 16	573.4	1724.3	**2.95**	< 0.0001
DUSP5	Dual specificity phosphatase 5	115.6	329.9	**2.79**	< 0.0001
SOD2	Superoxide dismutase 2	40215.6	112308.8	**2.77**	< 0.0001
TNFSF15	TNF superfamily member 15	50.5	6.0	**-5.98**	< 0.0001
CCL2	C-C motif chemokine ligand 2	176.3	29.9	**-5.43**	< 0.0001
FGFR3	Fibroblast growth factor receptor 3	1524.2	338.9	**-4.50**	< 0.0001
COX-2	Cyclooxygenase-2	1232.1	280.9	**-4.29**	< 0.0001
IL6	Interleukin 6	25.8	4.0	**-3.86**	< 0.0001
CX3CL1	C-X3-C motif chemokine ligand 1	30.1	10.6	**-2.58**	< 0.0001
IL11	Interleukin 11	30.4	8.2	**-2.58**	< 0.0001

Catabolic enzymes and their inhibitors

Gene	Name	Mean (Co)	Mean (D)	Fold Change	FDR *p*
MMP7	Matrix metalloproteinase 7	3.3	29.0	**4.89**	< 0.0001
MMP19	Matrix metalloproteinase 19	9.7	31.3	**2.89**	< 0.0001
MMP2	Matrix metalloproteinase 2	165.6	347.7	**2.06**	< 0.0001
MMP13	Matrix metalloproteinase 13	2452.4	536.6	**-4.08**	0.00034
MMP16	Matrix metalloproteinase 16	1004.9	323.5	**-3.12**	< 0.0001
MMP1	Matrix metalloproteinase 1	26580.9	9513.7	**-2.85**	< 0.0001
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif 9	484.4	1713.8	**3.14**	< 0.0001
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1	4476.2	1022.4	**-4.29**	< 0.0001
ADAMTS3	ADAM metallopeptidase with thrombospondin type 1 motif 3	207.5	70.6	**-2.83**	< 0.0001
TIMP4	TIMP metallopeptidase inhibitor 4	386.8	1717.0	**4.41**	< 0.0001
TIMP3	TIMP metallopeptidase inhibitor 3	14668.7	6429.5	**-2.35**	< 0.0001

Table 3 Selected genes linked to inflammation, oxidative stress, catabolic enzymes, and extracellular matrix production as well as growth factors in dexamethasone-treated OA chondrocytes (D) relative to control (Co) *(Continued)*

Extracellular matrix components

Gene	Name	Mean (Co)	Mean (D)	Fold Change	FDR*p*
COL13A1	Collagen type XIII alpha 1 chain	51.2	7.2	**-4.99**	< 0.0001
COL27A1	Collagen type XXVII alpha 1 chain	2220.9	468.6	**-4.76**	< 0.0001
COL9A1	Collagen type IX alpha 1 chain	678.5	176.2	**-3.66**	< 0.0001
COL1A1	Collagen type I alpha 1 chain	146.3	46.8	**-3.12**	< 0.0001
COL11A1	Collagen type XI alpha 1 chain	59852.0	19522.6	**-3.12**	< 0.0001
COL5A1	Collagen type V alpha 1 chain	5372.5	1790.5	**-2.95**	< 0.0001
COL14A1	Collagen type XIV alpha 1 chain	1474.2	562.4	**-2.69**	< 0.0001
COL16A1	Collagen type XVI alpha 1 chain	991.9	408.0	**-2.39**	< 0.0001
COL15A1	Collagen type XV alpha 1 chain	9376.8	4058.2	**-2.31**	< 0.0001
COL2A1	Collagen type II alpha 1 chain	130240.1	56195.3	**-2.28**	< 0.0001
COL5A2	Collagen type V alpha 2 chain	19215.6	8756.4	**-2.22**	< 0.0001
COL10A1	Collagen type X alpha 1 chain	2211.9	1059.0	**-2.16**	< 0.0001
COL1A2	Collagen type I alpha 2 chain	5477.4	2601.9	**-2.11**	< 0.0001
ACAN	Aggrecan	81094.4	202992.8	**2.43**	< 0.0001
ELN	Elastin	377.4	99.1	**-3.78**	< 0.0001

Growth factors

Gene	Name	Mean (Co)	Mean (D)	Fold Change	FDR*p*
TDGF1	Teratocarcinoma-derived growth factor 1	4.0	30.4	**5.21**	< 0.0001
CTGF	Connective tissue growth factor	16003.3	36823.5	**2.25**	< 0.0001
PGF	Placental growth factor	73.6	11.4	**-5.13**	< 0.0001
TGFB3	Transforming growth factor beta 3	318.8	63.1	**-4.86**	< 0.0001
NDP	NDP, norrin cystine knot growth factor	259.2	51.5	**-4.72**	< 0.0001
FGF1	Fibroblast growth factor 1	6015.9	2323.5	**-2.62**	< 0.0001
TGFB2	Transforming growth factor beta 2	7649.3	2938.2	**-2.57**	< 0.0001
VEGFA	Vascular endothelial growth factor A	8007.4	3366.9	**-2.36**	< 0.0001
GDF5	Growth differentiation factor 5	474.1	229.3	**-2.11**	< 0.0001

FDR*p* = False discovery rate -corrected *p*-value
Red = upregulated genes
Blue = downregulated genes

Discussion

The pathology of OA is characterized by eventual cartilage degradation that is caused by imbalanced gene expression profiles in the cartilage. The effects of GCs used in the treatment of OA on this balance are of interest, and RNA-Seq provides a comprehensive view of gene expression in a tissue. Dexamethasone was found to affect the expression of a large number of genes in OA chondrocytes. Among the most strongly affected genes were several involved in inflammation, extracellular matrix organization, and carbohydrate and lipid metabolism.

Constant low-grade joint inflammation along with inflammatory exacerbations is a central feature of OA. Glucocorticoids were found to inhibit inflammation, and this might explain part of their therapeutic effects on OA exacerbations. In our data, dexamethasone reduced the expression of well-known inflammatory factors such as cyclooxygenase-2 (COX-2) [38], interleukin 6 (IL6), and C-C motif chemokine ligand 2 (CCL2) [39]. In

a

Regulation of carbohydrate metabolism (GO:0006109)

Gene	Name	Mean (Co)	Mean (D)	Fold Change	FDR *p*
PDK4	Pyruvate dehydrogenase kinase 4	251.6	5804.9	**19.84**	< 0.0001
ADRA1B	Adrenoceptor alpha 1B	0.1	23.7	**14.72**	< 0.0001
KCNJ11	Potassium voltage-gated channel subfamily J member 11	0.3	21.6	**12.13**	< 0.0001
CLTCL1	Clathrin heavy chain like 1	104	693.2	**6.45**	< 0.0001
DCXR	Dicarbonyl and L-xylulose reductase	79	196.7	**2.41**	< 0.0001
PYGL	Glycogen phosphorylase L	1785.1	4258.4	**2.38**	< 0.0001
KCNB1	Potassium voltage-gated channel subfamily B member 1	21	54.5	**2.38**	0.003
IGFBP5	Insulin like growth factor binding protein 5	2688.8	435.7	**-6.07**	< 0.0001
SLC2A12	Solute carrier family 2 member 12	742.4	136.7	**-5.07**	< 0.0001
SOX4	SRY-box 4	1548.9	339.8	**-4.59**	< 0.0001
GALM	Galactose mutarotase	271.7	108.4	**-2.55**	< 0.0001

Regulation of lipid metabolism (GO:0019216)

Gene	Name	Mean (Co)	Mean (D)	Fold Change	FDR *p*
ABCD2	ATP binding cassette subfamily D member 2	13.8	340.3	**18.77**	< 0.0001
ABCA6	ATP binding cassette subfamily A member 6	102.4	1070.3	**9.99**	< 0.0001
SORBS1	Sorbin and SH3 domain containing 1	648.8	4163.1	**6.36**	< 0.0001
APOD	Apolipoprotein D	638.3	3819.4	**6.15**	< 0.0001
APOB	Apolipoprotein B	15.7	95.5	**5.16**	< 0.0001
PRKAG2	Protein kinase AMP-activated non-catalytic subunit gamma 2	470.8	1591.1	**3.29**	< 0.0001
PPARG	Peroxisome proliferator activated receptor gamma	24.5	79.5	**3.12**	< 0.0001
SESN3	Sestrin 3	4701.2	14511	**3.1**	< 0.0001
FABP4	Fatty acid binding protein 4	124.8	374.2	**2.93**	< 0.0001
IRS2	Insulin receptor substrate 2	364.5	902.6	**2.39**	< 0.0001
PLIN2	Perilipin 2	875.4	1834.3	**2.07**	< 0.0001
FABP3	Fatty acid binding protein 3	624.2	241.1	**-2.58**	< 0.0001
FABP5	Fatty acid binding protein 5	1357.8	634.5	**-2.13**	< 0.0001

FDR *p* = False discovery rate -corrected *p*-value, Red = upregulated genes, Blue = downregulated genes

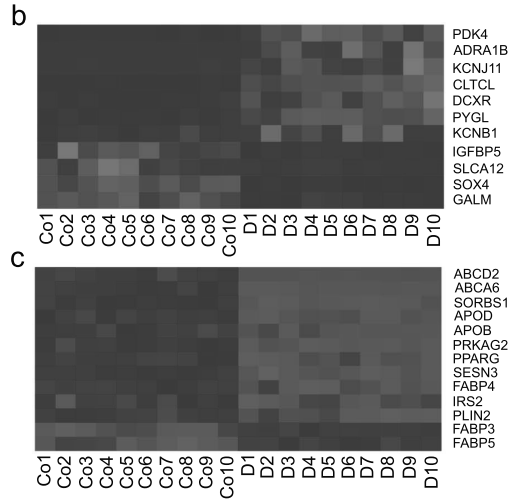

Fig. 1 Genes significantly affected by dexamethasone and regulating carbohydrate (GO:0006109) and lipid (GO:0019216) metabolism. **a** Table showing the expression of genes regulating carbohydrate and lipid metabolism in dexamethasone-treated OA chondrocytes (D) compared with control (Co). **b** and **c** Heatmaps showing the expression of differentially expressed genes involved in carbohydrate and lipid metabolism, respectively, in dexamethasone-treated OA cells (D) and controls (Co). Expression values are DESeq2-normalized and row-scaled, with blue signifying lower and red higher expression

addition, dexamethasone downregulated the expression of cartilage extracellular matrix-degrading matrix metalloproteinases (MMPs) 1, 13, and 16, while various collagens and anabolic factors (including hyaluronan synthase 2 [HAS2], one of the most strongly downregulated genes) were also downregulated. As the relative expression of catabolic and anabolic factors in the cartilage varies during the course of OA, the effects of glucocorticoids on cartilage homeostasis are likely to depend on the phase of the disease process.

Osteoarthritis as well as glucocorticoid treatment has been reported to be associated with increased oxidative stress and chondrocyte apoptosis [18]. Activation of the ROS/Akt/FOXO3 signaling pathway appears to counteract these effects, and particularly, forkhead box O3 (FOXO3) has been shown to protect chondrocytes from apoptosis [40]. Interestingly, the expression of FOXO3 was strongly upregulated by dexamethasone in the present data. Superoxide dismutase 2 (SOD2) inactivates the superoxide anion, reducing oxidative stress [21]. SOD2 was highly expressed in OA cartilage, and its expression was nearly tripled by treatment with dexamethasone. On the other hand, dexamethasone strongly enhanced the expression of KLF9, which has been shown to sensitize cells to oxidative stress [41] and may contribute to the previously reported dexamethasone-induced oxidative stress [18].

Pain in OA is mediated through various intracellular pathways and soluble factors, of which nerve growth factor (NGF) is thought to be of particular importance [42]. Antibodies targeting NGF have been shown to be effective for treating OA pain but may also accelerate joint destruction in a small group of patients [36]. In the present data, the expression of NGF was reduced by dexamethasone indicating that intra-articular GC injections might alleviate OA pain at least partly by reducing the synthesis of NGF. The mechanisms of NGF blocker-induced joint degradation are still largely unknown. Increased use of the OA-affected joint, enabled by analgesia, has been hypothesized to explain the findings. However, accelerated degradation has been observed also in non-OA-affected (and initially painless) joints, casting doubt on this hypothesis [36]. Whether GC-induced downregulation of NGF might have similar deleterious effects remains to be studied. In addition to NGF, dexamethasone treatment also downregulated the expression of prostaglandin-producing COX-2 and vascular endothelial growth factor A (VEGFA). Prostaglandins, particularly PGE_2, as well as VEGFA are involved in mediating OA pain [43]. Thus, their downregulation by dexamethasone is likely to contribute to the pain-alleviating properties of glucocorticoids in OA.

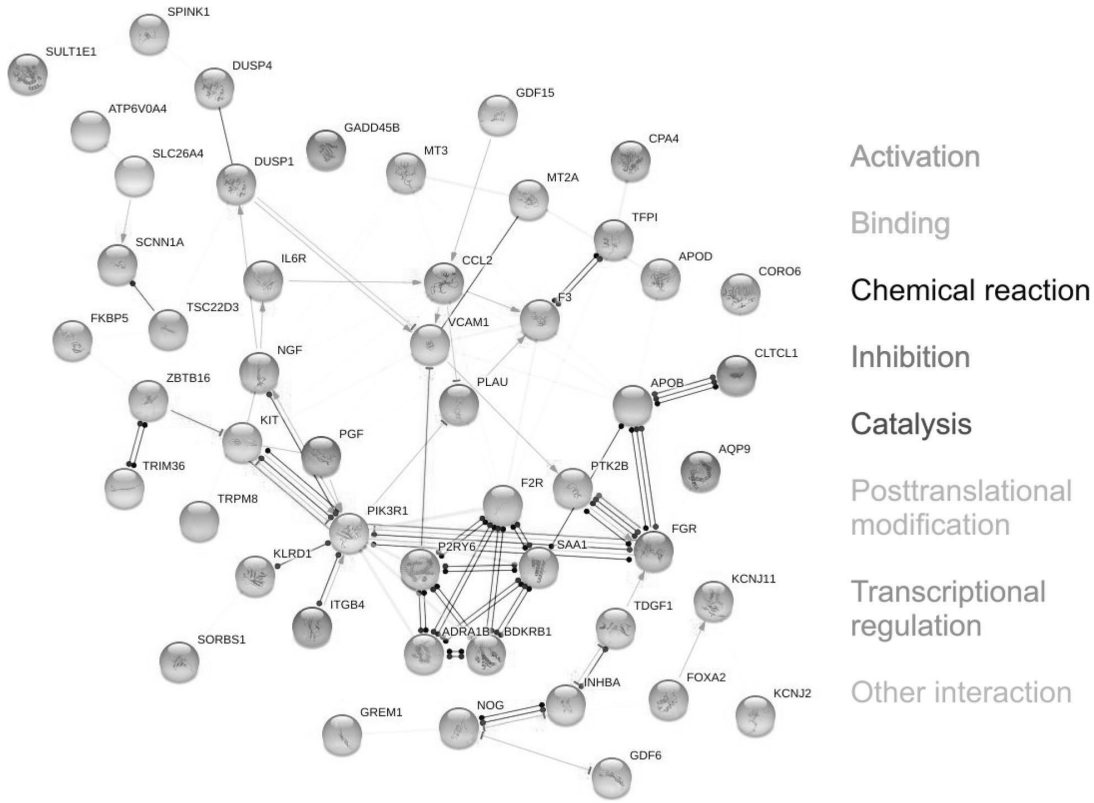

Fig. 2 Interactions among the genes that were up- or downregulated by dexamethasone with an FC 5.0 or greater into either direction, as determined with STRING. Genes with no interactions are excluded from the graph. Colors of the edges are as follows: green = activation, blue = binding, black = chemical reaction, red = inhibition, violet = catalysis, pink = posttranslational modification, yellow = transcriptional regulation, and gray = other interaction

Dexamethasone markedly affected the expression of several genes involved in glucose and lipid metabolism. An interesting example is pyruvate dehydrogenase kinase 4 (PDK4), which was one of the genes most strongly upregulated by dexamethasone. As this gene inactivates pyruvate dehydrogenase and prevents pyruvate produced in glycolysis from progressing to oxidative phosphorylation [44], this glucocorticoid effect may shift carbohydrate metabolism from mitochondrial respiration towards glycolysis. Several genes promoting lipid synthesis and transport, such as perilipin 2 (PLIN2) [45] and 5′-AMP-activated protein kinase subunit gamma-2 (PRKAG2) [46], were also upregulated. However, genes coding for the enzymes participating in the major pathways of carbohydrate and lipid metabolism itself (glycolysis, oxidative phosphorylation, lipolysis, and beta-oxidation) were not significantly affected. Of the OA-associated lipid metabolism genes, apolipoprotein D (APOD) is a central mediator of steroid and lipoprotein metabolism. Its expression has been shown to be decreased in OA chondrocytes [47], and APOD was found to be upregulated by dexamethasone in the present study. Sestrin 3 (SESN3), which was highly expressed in OA cartilage and upregulated by dexamethasone, is involved in the regulation of carbohydrate and lipid metabolism. Impairment of Sestrin signaling has been implicated in the pathogenesis of OA [48]. These are examples of dexamethasone-induced normalization of the expression of lipid metabolism-related genes in OA chondrocytes. Furthermore, peroxisome proliferator-activated receptor gamma (PPARG), which was likewise upregulated by dexamethasone, is widely regarded as an anti-inflammatory and chondroprotective factor [49] in addition to its significant role in cellular metabolism.

Osteoarthritis has a large heritable component, as up to 50% of the incidence of the disease is thought to be explained by genetics [50, 51]. Several genome-wide association (GWAS) studies have been performed in OA [23–25], identifying at least 53 genes associated with the

disease. Many of the identified genes affect extracellular matrix synthesis and skeletal system development, while the effects of some of the genes are largely unknown [23–25]. GWAS studies investigate systemic genomic variation, while expression analyses (such as the present study) directly measure gene expression in the tissue of interest. Nevertheless, as gene polymorphisms often affect the function or activity of the protein coded by the gene, it can be postulated that altered expression or activity of the OA-linked genes might affect the development of the disease and serve as a treatment target. Twelve of the 53 genes previously associated with hip and/or knee OA in GWAS studies were found to be clearly (FC > 2.0) affected by dexamethasone. Examples of those are COL11A1, COX-2, GDF5, IL6, and VEGFA, all of which were downregulated. Our study identified hundreds of differential genes by dexamethasone, which makes it quite probable that some of them should, by chance, be among those previously linked to OA. However, in the case of those genes which have relatively well-established mechanistic links to OA development, we think that highlighting them as genes potentially mediating the effects of glucocorticoids on the development or symptoms of the disease is justified. For example, GDF5 may affect cartilage remodeling and repair [52], COX-2 and IL6 are indicated to promote inflammation in OA [39], and VEGF-induced angiogenesis seems to play a role in OA progression and pain [42]. Further elucidating the relative glucocorticoid-regulated effects of these genes on OA pathophysiology could be an interesting avenue of further study.

The microarray-based genome-wide expression analysis (GWEA) study by Ramos et al. [9] previously compared the gene expression in OA affected and preserved cartilage in the same joint. It identified 18 differentially expressed genes, whose up- or downregulation might therefore be expected to affect the pathogenesis of OA. Two of those, namely NGF and collagen 9 alpha 1 (COL9A1), were markedly affected by dexamethasone in the present study. NGF was upregulated in more severely affected OA cartilage [9], and in the present study, dexamethasone was found to downregulate it. As previously discussed, NGF downregulation might alleviate OA pain but also predispose the cartilage to accelerated destruction [36]. COL9A1 was downregulated in severely affected OA cartilage [9], and dexamethasone further downregulated its expression in our data. This can be regarded as an antianabolic effect with an impact on OA, which is further supported by the finding that COL9A1 deficiency induces osteoarthritis-like pathology in mice [53]. The results of our study were also compared with a recent larger, NGS-based expression analysis based on an extended study population [10]. Of the 372 genes identified in that study with markedly differential expression (FC > 2.0 in either direction) between degraded and preserved OA cartilage, 78 were significantly affected by dexamethasone in our study. While the expression of 42 genes was "normalized" by dexamethasone (i.e., their expression was altered in the direction of preserved cartilage), nearly the same number (34) were altered in the opposite direction. Thus, while glucocorticoids may partially normalize the phenotype of OA chondrocytes, this may be counteracted by increased expression of genes driving the disease process in OA cartilage.

The time course of the effects of glucocorticoids is an important factor to be taken into account when evaluating their potential effects on cartilage. When used at clinically relevant doses (1–3 mg), dexamethasone injected intra-articularly seems to be mostly absorbed from the joint within 24 h [54]. We thus propose that the time point used in the current study (24 h) can be expected to reasonably well capture the effects of glucocorticoids on gene expression in OA chondrocytes, while the effects on protein production are known to occur and last over a longer time frame (several days) [55]. Investigating the time evolution of chondrocyte gene expression in response to glucocorticoid treatment (including direct and secondary effects) would be an interesting avenue of further study.

Conclusions

In conclusion, dexamethasone was found to cause a major phenotypic switch in OA chondrocytes, while the overall effect on genes linked to OA in GWAS and GWEA studies appeared to be modest. In addition to clear anti-inflammatory, anticatabolic, and extracellular matrix-targeting effects, dexamethasone was found to affect lipid and glucose metabolism-related genes, an observation that might be particularly important in the metabolic phenotype of OA.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13075-020-02289-7.

Additional file 1: Table S1. Primers and probes used for quantitative RT-PCR. **Table S3.** Expression of cartilage constituents in dexamethasone-treated OA chondrocytes (D) relative to controls (Co). **Table S4.** Selected genes linked to inflammation, oxidative stress, catabolism and extracellular matrix production in dexamethasone-treated OA chondrocytes (D) relative to controls (Co) as determined by NGS and confirmed with RT-PCR. **Table S5.** Expression of genes belonging to the major pathways of carbohydrate and lipid metabolism in dexamethasone-treated OA chondrocytes (D) relative to controls (Co) [35]. **Table S6.** Effects of dexamethasone on genes linked to OA in previous GWAS studies [21–23]. **Table S7.** Effects of dexamethasone on genes previously linked to OA in the GWEA study by Ramos et al. [9]. **Table S8.** Effects of dexamethasone on genes previously linked to OA cartilage in the GWEA study by Almeida et al. [10]. **Figure S1.** Effects of dexamethasone on the production of catabolic and proinflammatory factors in OA chondrocytes. OA chondrocytes / chondrocytes isolated from OA patients were cultured for 24 h with or without dexamethasone (1 μM). MMP-1 (A), MMP-13 (B) and CCL2 (C) levels in the culture media were determined with ELISA. MMP-1 (D), MMP-13 (E) and CCL2 (F) mRNA expression

was studied with quantitative RT-PCR and normalized against GAPDH. The results were compared against control, which was set as 100 %. The results are expressed as mean + SEM, $n = 9$. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$, compared to the untreated control.

Additional file 2: Table S2. All genes differentially expressed in dexamethasone-treated OA chondrocytes (D) relative to controls (Co).

Abbreviations

ECM: Extracellular matrix; ELISA: Enzyme-linked immunosorbent assay; FC: Fold change; FDR: False discovery rate; GC: Glucocorticoid; GO: Gene Ontology; GWEA: Genome-wide expression analysis; GWAS: Genome-wide association study; MMP: Matrix metalloproteinase; mRNA: Messenger RNA; NGS: Next-generation sequencing; OA: Osteoarthritis; RNA-Seq: RNA sequencing; RT-PCR: Real-time quantitative polymerase chain reaction; SEM: Standard error of the mean

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Authors' contributions

AP was involved in the conception and design of the study and in the laboratory analyses; he analyzed the data and drafted the manuscript. TL was involved in the design of the study, in the laboratory analyses, in the interpretation of data, and in revising the manuscript. MH was involved in the design of the study, in the laboratory analyses, and in revising the manuscript. TM was involved in the design of the study, in selecting the patients and acquiring patient samples, in the interpretation of the data, and in revising the manuscript. KV was involved in the design of the study, in the interpretation of the data, and in revising the manuscript. EM supervised the study being particularly involved in the conception and design of the study, in the interpretation of the data, and in writing the manuscript. All authors approved the final version of the manuscript.

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Availability of data and materials

The list of all genes significantly affected by dexamethasone is included in Supplementary data (Table S2).

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Tampere University Hospital, Finland, and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Effects of ibuprofen on gene expression in chondrocytes from patients with osteoarthritis as determined by RNA-Seq

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SHORT REPORT

Effects of ibuprofen on gene expression in chondrocytes from patients with osteoarthritis as determined by RNA-Seq

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ABSTRACT

Non-steroidal anti-inflammatory drugs are a widely used symptomatic treatment in osteoarthritis (OA), but their effects on cartilage remain controversial. We studied the effects of ibuprofen on gene expression in chondrocytes from patients with OA using RNA-Seq. Chondrocytes were isolated from cartilage samples of patients with OA undergoing knee replacement surgery, cultured with ibuprofen, and total mRNA was sequenced. Differentially expressed genes were identified with edgeR using pairwise comparisons. Functional analysis was performed using ingenuity pathway analysis (IPA). Ibuprofen did not induce statistically significant changes in chondrocyte transcriptome when the cells were cultured in the absence of added cytokines. In inflammatory conditions (when the cells were exposed to the OA-related cytokine interleukin (IL)-1β), 51 genes were upregulated and 42 downregulated by ibuprofen with fold change >1.5 in either direction. The upregulated genes included anti-inflammatory factors and genes associated with cell adhesion, while several mediators of inflammation were among the downregulated genes. IPA analysis revealed ibuprofen having modulating effects on inflammation-related pathways such as integrin, IL-8, ERK/MAPK and cAMP-mediated signalling pathways. In conclusion, the effects of ibuprofen on primary OA chondrocyte transcriptome appear to be neutral in normal conditions, but ibuprofen may shift chondrocyte transcriptome towards anti-inflammatory phenotype in inflammatory environments.

Key messages

- The current evidence about the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on osteoarthritis (OA) cartilage is conflicting.
- We investigated the effects of ibuprofen on gene expression in OA chondrocytes by using RNA-Seq.
- In neutral conditions (in the absence of added inflammatory factors), ibuprofen had no statistically significant effects on gene expression in OA chondrocytes.
- In inflammatory conditions mimicked by the presence of interleukin (IL)-1β, ibuprofen upregulated several anti-inflammatory factors while downregulating inflammatory mediators such as IL-6 and IL-23. Ibuprofen also inhibited phosphatase and tensin homolog (PTEN) signalling.
- The findings support the assumption that NSAIDs are safe for cartilage when treating OA pain. They also may shift chondrocyte transcriptome towards an anti-inflammatory phenotype in OA exacerbations.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat osteoarthritis (OA) pain but there are some concerns on their effects on chondrocyte biology.[1]

OA is characterised by constant low-grade joint inflammation and transient inflammatory exacerbations. The inflammatory nature of the disease is evidenced by the increased production of proinflammatory cytokines, particularly interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor α (TNFα). They drive the production of catabolic enzymes such as matrix metalloproteinases (MMPs), accelerating joint destruction.[2]

NSAIDs exert their effects by inhibiting the synthesis of prostanoids, particularly prostaglandin E_2 (PGE_2) by cyclo-oxygenase (COX) enzymes. By altering the balance of proinflammatory and anti-inflammatory mediators in the joint, they have been hypothesised to affect OA pathogenesis. These effects, if any, are however controversial, as both potential benefits (eg, alleviation of joint inflammation and reduction of cartilage catabolism) and harms (eg, impairment of cartilage anabolism and accelerated radiographic joint destruction) have been reported.[1 3]

We carried out a genome-wide expression analysis on the effects of the NSAID ibuprofen on gene expression in OA chondrocytes in

Table 1 All genes upregulated or downregulated by ibuprofen in the presence of IL-1β with FC ≥1.5 in either direction

Gene	Name	Function	Mean (IL1)	Mean (IL1 +ibu)	FC	adj. P
PPARG	Peroxisome proliferator activated receptor gamma	Carbohydrate and lipid metabolism, inflammation	0.3	0.9	**2.87**	5.0E−06
UMODL1	Uromodulin like 1	Regulation of apoptosis?	0.3	0.7	**2.39**	0.0011
XIRP1	Xin actin binding repeat containing 1	Actin binding	5.7	13.4	**2.38**	< 1.0E−06
DACT1	Dishevelled binding antagonist of beta catenin 1	Regulation of cell cycle and tissue development	4	8.4	**2.1**	< 1.0E−06
CSF2/GM-CSF	Colony stimulating factor 2=Granulocyte-macrophage colony stimulating factor	Leucocyte differentiation, immune response	5.2	11.2	**2.09**	< 1.0E−06
PPARGC1B	PPARG coactivator 1 beta	Regulation of transcription	0.4	0.8	**2.07**	0.00024
FAM186B	Family with sequence similarity 186 member B	?	0.4	0.7	**1.92**	0.0035
SOX17	SRY-box 17	Cell proliferation, tissue development	3.5	6.8	**1.91**	< 1.0E−06
MTSS1	MTSS1, I-BAR domain containing	Cell adhesion	6.9	12.6	**1.9**	< 1.0E−06
AKAP6	A-kinase anchoring protein 6	Regulation of cell proliferation, cAMP signalling	2.1	3.9	**1.89**	< 1.0E−06
PDE5A	Phosphodiesterase 5A	Regulation of NO signalling	1.2	2.3	**1.85**	7.0E−06
RGS2	Regulator of G protein signalling 2	Regulation of G protein signalling	52.1	95.9	**1.85**	< 1.0E−06
CAMK2A	Calcium/calmodulin dependent protein kinase II alpha	Wnt and TGFβ signalling, NF-κB activation	1.8	3.3	**1.81**	< 1.0E−06
MAP1LC3C	Microtubule associated protein 1 light chain 3 gamma	Autophagy	0.7	1.2	**1.79**	3.3E−05
NRG1	Neuregulin 1	Cell differentiation, signal transduction	0.7	1.3	**1.78**	5.9E−05
SELE	Selectin E	Inflammation	41.3	73.5	**1.78**	< 1.0E−06
FCRLA	Fc receptor like A	Immunoglobulin binding	4.2	7.4	**1.76**	< 1.0E−06
DENND3	DENN domain containing 3	Autophagy	14.6	25.3	**1.75**	< 1.0E−06
FCRLB	Fc receptor like B	Immunoglobulin binding	0.8	1.4	**1.73**	3.3E−05
MOXD1	Monooxygenase DBH like 1	Monoamine metabolism	123.1	210.3	**1.72**	< 1.0E−06
SPNS2	Sphingolipid transporter 2	Lipid transport	2.4	4.0	**1.72**	< 1.0E−06
PODXL	Podocalyxin like	Cell adhesion	11.2	19.0	**1.71**	< 1.0E−06
RP1	RP1, axonemal microtubule associated	?	0.4	0.6	**1.67**	0.023
IDO1	Indoleamine 2,3-dioxygenase 1	Modulation of inflammation and cartilage development	1.3	2.1	**1.65**	0.0012
SCUBE3	Signal peptide, CUB domain and EGF like domain containing 3	TGFβ signalling	42.5	72.5	**1.64**	< 1.0E−06
KCNJ15	Potassium voltage-gated channel subfamily J member 15	Potassium transport	1.3	2.2	**1.63**	1.0E−06
SERPINE1	Serpin family E member 1	Inhibition of proteolysis	455.9	748.6	**1.62**	< 1.0E−06
PSD2	Pleckstrin and Sec7 domain containing 2	?	0.4	0.6	**1.62**	0.035
LINGO1	Leucine rich repeat and Ig domain containing 1	?	1.9	3.1	**1.61**	< 1.0E−06
AKNAD1	AKNA domain containing 1	?	0.6	1.0	**1.60**	0.0048
STRA6	Stimulated by retinoic acid 6	Retinol and adipokine binding	1.9	2.9	**1.59**	0.00058
ITGAX	Integrin subunit alpha X	Cell adhesion	11.0	17.4	**1.58**	< 1.0E−06
KCNN3	Potassium calcium-activated channel subfamily N member 3	Potassium transport	2.8	4.3	**1.58**	< 1.0E−06
ICAM5	Intercellular adhesion molecule 5	Cell adhesion	4.5	7.2	**1.58**	< 1.0E−06
FGD4	FYVE, RhoGEF and PH domain containing 4	Cytoskeleton organisation	21.9	34.0	**1.57**	< 1.0E−06
KCNN4	Potassium calcium-activated channel subfamily N member 4	Potassium transport	6.4	10.0	**1.57**	< 1.0E−06
LRRC55	Leucine rich repeat containing 55	Potassium transport	0.8	1.3	**1.56**	0.0013
CXCR3	C-X-C motif chemokine receptor 3	Inflammation	0.7	1.1	**1.55**	0.017
CD24	CD24 molecule	Wnt and MAPK signalling, regulation of inflammation	5.4	8.5	**1.55**	< 1.0E−06
FGR	FGR proto-oncogene, Src family tyrosine kinase	PI3K-Akt signalling, regulation of inflammation	3.4	5.2	**1.54**	< 1.0E−06

Continued

Table 1 Continued

Gene	Name	Function	Mean (IL1)	Mean (IL1 +ibu)	FC	adj. P
PEG10	Paternally expressed 10	Inhibition of TGFβ signalling	23.9	36.4	**1.54**	< 1.0E–06
SIGLEC15	Sialic acid binding Ig like lectin 15	Regulation of bone resorbtion	1.6	2.4	**1.54**	0.00024
CPNE2	Copine 2	Bone erosion	18.7	28.9	**1.54**	< 1.0E–06
WNK4	WNK lysine deficient protein kinase 4	Ion transport	4.6	7.0	**1.53**	< 1.0E–06
RTL3	Retrotransposon Gag like 3	Regulation of collagen production	3.3	5.0	**1.53**	< 1.0E–06
RGS3	Regulator of G protein signalling 3	Inhibition of MAPK signalling	65.1	99.2	**1.52**	< 1.0E–06
AOC2	Amine oxidase, copper containing 2	Amine metabolism	68.0	102.3	**1.51**	< 1.0E–06
IL10RA	Interleukin 10 receptor subunit alpha	Regulation of inflammation	1.0	1.6	**1.51**	0.0018
RGS16	Regulator of G protein signalling 16	?	60.1	90.0	**1.51**	< 1.0E–06
PCDH17	Protocadherin 17	Cell adhesion	0.9	1.4	**1.51**	0.028
GPR158	G protein-coupled receptor 158	?	1.3	1.9	**1.50**	0.00017
IL23A	Interleukin 23 subunit alpha	Inflammation	15.2	4.7	**–3.24**	< 1.0E–06
HAS1	Hyaluronan synthase 1	Extracellular matrix production	0.8	0.3	**–2.77**	< 1.0E–06
IGFBP4	Insulin-like growth factor binding protein 4	Cell proliferation and metabolism	213.8	79.7	**–2.73**	< 1.0E–06
IL6	Interleukin 6	Inflammation	958.4	403.8	**–2.49**	< 1.0E–06
PDE3A	Phosphodiesterase 3A	Lipid metabolism	0.9	0.3	**–2.48**	0.00013
STAT4	Signal transducer and activator of transcription 4	Inflammation, regulation of cell proliferation	2.5	1.0	**–2.36**	< 1.0E–06
PCSK1	Proprotein convertase subtilisin/kexin type 1	Metabolism	7.2	3.2	**–2.19**	< 1.0E–06
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif 6	Extracellular matrix catabolism	10.5	4.9	**–2.18**	< 1.0E–06
HAL	Histidine ammonia-lyase	Histidine catabolism	1.7	0.8	**–2.12**	< 1.0E–06
DNAH17	Dynein axonemal heavy chain 17	Cytoskeleton component	1.0	0.5	**–2.06**	2.00E–06
CSF3	Colony stimulating factor 3	Inflammation, regulation of cell proliferation	19.8	9.9	**–2.02**	< 1.0E–06
AREG	Amphiregulin	EGF signalling, regulation of cell proliferation	2.3	1.2	**–2.01**	< 1.0E–06
CA12	Carbonic anhydrase 12	Acidity regulation, Regulation of proliferation	20.9	10.5	**–2.00**	< 1.0E–06
INSC	Inscuteable homolog (Drosophila)	Cell differentiation	0.6	0.3	**–1.98**	0.0011
KCNE5	Potassium voltage-gated channel subfamily E regulatory subunit 5	Regulation of potassium transport	1.3	0.6	**–1.94**	6.00E–06
LDB2	LIM domain binding 2	Regulation of transcription	0.5	0.3	**–1.92**	0.005098
DOK6	Docking protein 6	?	0.9	0.5	**–1.80**	0.000598
DAW1	Dynein assembly factor with WD repeats 1	Dynein assembly	0.9	0.5	**–1.78**	0.000565
TMEM71	Transmembrane protein 71	?	1.8	1.0	**–1.77**	2.00E–06
MAMSTR	MEF2 activating motif and SAP domain containing transcriptional regulator	Regulation of transcription	0.5	0.3	**–1.72**	0.021819
KNDC1	Kinase non-catalytic C-lobe domain containing 1	?	0.8	0.5	**–1.70**	0.002773
EFHC2	EF-hand domain containing 2	Cell proliferation	0.8	0.5	**–1.69**	0.004747
MEX3A	Mex-3 RNA binding family member A	PI3K-Akt signalling	0.9	0.5	**–1.69**	0.001905
TGFBI	Transforming growth factor beta induced	ECM organisation, chondrocyte differentiation	127.8	80.7	**–1.64**	< 1.0E–06
C3AR1	Complement C3a receptor 1	Inflammation	3.5	2.2	**–1.63**	< 1.0E–06
EFEMP1	EGF containing fibulin like extracellular matrix protein 1	Inhibition of chondrocyte differentiation	72.6	45.4	**–1.63**	< 1.0E–06
NAMPT	Nicotinamide phosphoribosyltransferase / visfatin	Cartilage catabolism	596.1	368.8	**–1.60**	< 1.0E–06
FOXF1	Forkhead box F1	Morphogenesis	1.2	0.8	**–1.60**	0.000928
AVPI1	Arginine vasopressin induced 1	MAPK signalling	39.7	24.8	**–1.60**	< 1.0E–06
SEMA3A	Semaphorin 3A	Regulation of inflammation and apoptosis	98.0	61.6	**–1.59**	< 1.0E–06
STC1	Stanniocalcin 1	Regulation of cartilage development	2.0	1.3	**–1.59**	0.002967

Continued

Table 1 Continued

Gene	Name	Function	Mean (IL1)	Mean (IL1 +ibu)	FC	adj. P
TSKU	Tsukushi, small leucine rich proteoglycan	?	14.9	9.4	**−1.58**	< 1.0E−06
SMOC1	SPARC related modular calcium binding 1	ECM organisation	199.0	126.1	**−1.57**	< 1.0E−06
ARAP2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	Cell adhesion, inhibition of Akt signalling	12.5	8.0	**−1.56**	< 1.0E−06
BEND5	BEN domain containing 5	Negative regulation of transcription	0.9	0.6	**−1.54**	0.01673
EPB41L3	Erythrocyte membrane protein band 4.1 like 3	Cortical cytoskeleton organisation	34.6	22.5	**−1.53**	< 1.0E−06
EPB41L4B	Erythrocyte membrane protein band 4.1 like 4B	Regulation of cell adhesion and gene expression	2.8	1.8	**−1.53**	0.000106
ACSL4	Acyl-CoA synthetase long-chain family member 4	Lipid metabolism	428.1	280.7	**−1.52**	< 1.0E−06
NR4A2	Nuclear receptor subfamily 4 group A member 2	Wnt signalling, regulation of apoptosis	19.8	13.1	**−1.51**	< 1.0E−06
PID1	Phosphotyrosine interaction domain containing 1	Oxidative metabolism	285.1	190.6	**−1.51**	< 1.0E−06
RSPO3	R-spondin 3	Wnt signalling, morphogenesis	82.9	53.8	**−1.50**	< 1.0E−06

Red = upregulated genes; blue = downregulated genes.
adj. P, False discovery rate (FDR) -adjusted P value; IL, interleukin; Mean, trimmed mean of M-values (TMM) normalized counts.

normal and inflammatory conditions in vitro by using RNA-Seq.

METHODS

Cartilage samples were obtained from 10 patients with OA (mean age 67 years (SEM 3.8 years), 8 females, Kellgren-Lawrence grade 3.7 (SEM 0.15)) undergoing knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland.

Chondrocytes were isolated by enzyme digestion and seeded on 24-well plates for 24 hours. Thereafter the experiments were started, and the cells were cultured either alone, with ibuprofen (10 µM), with IL-1β (100 pg/mL), or with a combination of ibuprofen and IL-1β for 24 hours. Cell culture, RNA sequencing, RT-PCR and data analysis are described in online supplemental data S1.

RESULTS

The effects of ibuprofen on OA chondrocytes in neutral conditions

In the absence of exogenous cytokines, no genes were found to be differentially expressed between chondrocytes cultured with or without ibuprofen when the results were adjusted by false discovery rate.

The effects of ibuprofen on OA chondrocytes in inflammatory conditions

In inflammatory conditions (ie, in the presence of the OA-related cytokine IL-1β), ibuprofen induced the upregulation of 51 genes while 42 were downregulated in a statistically significant manner with a fold change >1.5 into either direction (table 1). All differentially expressed genes are listed in online supplemental tables S2 and S3.

The upregulated genes included anti-inflammatory factors such as peroxisome proliferator-activated receptor gamma (*PPARG*) and its coactivator *PPARGC1B* as well as IL-10 receptor subunit alpha. In addition, some genes associated with inflammation, including C-X-C motif chemokine receptor 3 (*CXCR3*), selectin E (*SELE*) and granulocyte-macrophage colony stimulating factor (*CSF2*/GM-CSF) were also upregulated (table 1).

On the other hand, several mediators of inflammation (such as *IL23A, IL6* and *NAMPT* (nicotinamide phosphoribosyltransferase aka visfatin)) were downregulated, as was the catabolic enzyme *ADAMTS6* (ADAM metallopeptidase with thrombospondin type 1 motif 6). Insulin-like growth factor-binding protein 4 (*IGFBP4*), which sequesters IGF and regulates chondrocyte proliferation,[4] was also downregulated. Hyaluronan synthase 1 (*HAS1*) and stanniocalcin-1 (*STC1*), previously shown to be upregulated in inflamed OA synovium,[5] were also downregulated by ibuprofen (table 1).

Differential expression of selected inflammation and cartilage-related genes (*PPARG*, *PPARGC1B*, *CSF2*, *IL23*, *HAS1*, *IGFBP4*, *ADAMTS6* and *IL6*) was confirmed with RT-PCR using chondrocytes from a different set of 10 patients (online supplemental figure S4). As expected, IL-1β was shown to strongly increase the synthesis of prostanoids, and this increase was inhibited by ibuprofen (online supplemental figure S5).

When all genes affected by ibuprofen in a statistically significant manner in the presence of IL-1β were analysed with ingenuity pathway analysis (IPA), activated canonical pathways included several associated with inflammation and cell adhesion such as IL-8, integrin, ERK/MAPK and cAMP-mediated signalling pathways (table 2). Conversely, phosphatase and tensin homolog (PTEN) signalling was inhibited (table 2). Differentially expressed genes included in the significantly activated/inhibited pathways are listed in online supplemental table S6.

Table 2 Canonical IPA pathways significantly upregulated or downregulated (z-score ≥2.5 or ≤–2.5) by ibuprofen in the presence of IL-1β

Canonical pathway	adj. P	z-score
Integrin signalling	4.37E–08	**4.95**
Actin cytoskeleton signalling	0.0022	**4.24**
PI3K signalling in B lymphocytes	0.00032	**3.44**
Agrin Interactions at neuromuscular junction	0.0037	**3.32**
IL-8 signalling	7.08E–07	**3.29**
ERK5 signalling	0.0083	**3.16**
Glioblastoma multiforme signalling	1.32E–06	**3.14**
Paxillin signalling	4.27E–06	**3.05**
ErbB2-ErbB3 signalling	0.029	**3.00**
FcγRIIB signalling in B lymphocytes	0.025	**3.00**
Renal cell carcinoma signalling	0.0016	**3.00**
Bladder cancer signalling	6.31E–06	**3.00**
14-3-3-mediated signalling	0.0058	**2.89**
PKCθ signalling in T lymphocytes	0.030	**2.84**
Calcium signalling	0.0083	**2.84**
Thrombin signalling	0.0025	**2.83**
CREB signalling in neurons	0.0019	**2.83**
HGF signalling	1.15E–06	**2.83**
Non-small cell lung cancer signalling	0.0029	**2.83**
α-Adrenergic signalling	5.37E–06	**2.71**
Endothelin-1 signalling	0.0098	**2.68**
Mouse embryonic stem cell pluripotency	0.0052	**2.67**
NF-κB activation by viruses	0.00089	**2.67**
Macropinocytosis signalling	4.27E–07	**2.67**
CXCR4 signalling	0.0048	**2.67**
p70S6K signalling	0.0026	**2.67**
cAMP-mediated signalling	0.0034	**2.56**
ErbB4 signalling	0.014	**2.53**
Chemokine signalling	0.013	**2.53**
Actin nucleation by ARP-WASP complex	0.00078	**2.53**
Regulation of cellular mechanics by calpain protease	5.25E–05	**2.53**
Synaptic long-term potentiation	0.00011	**2.52**
Cardiac hypertrophy signalling	0.00015	**2.50**
ERK/MAPK signalling	1.91E–05	**2.50**
fMLP signalling in neutrophils	0.0012	**2.50**
PAK signalling	0.00013	**2.50**
Rac signalling	0.026	**2.50**
IL-3 signalling	0.0018	**2.50**
Acute myeloid leukaemia signalling	0.0017	**2.50**
Telomerase signalling	0.0011	**2.50**
Wnt/Ca+pathway	5.25E–05	**2.50**
PTEN signalling	0.00087	**–2.67**

adj.P, False discovery rate (FDR) -adjusted P value; CREB, cAMP response element-binding protein; IL-1β, interleukin 1β; IPA, ingenuity pathway analysis.

Among the genes with FC >1.5 in either direction, STRING analysis identified *IL6* (which was downregulated by ibuprofen) as a central node in the interaction network (figure 1). Other genes occupying central places include PPARG, granulocyte-macrophage colony-stimulating factor and selectin E (*PPARG*, *CSF2* and *SELE* respectively, all upregulated by ibuprofen).

DISCUSSION

Ibuprofen did not have any significant effects on gene expression in primary OA chondrocytes cultured in the absence of added cytokines. This implies that ibuprofen has a neutral effect on chondrocyte transcriptome in non-inflamed joints. In cells treated with IL-1β, ibuprofen regulated the expression of both proinflammatory and anti-inflammatory factors and seemed to shift the balance to favour the latter.

Ibuprofen is a widely used non-selective NSAID. Like other NSAIDs, it exerts its effects by inhibiting prostanoid, particularly PGE_2, synthesis by COX-1 and COX-2 enzymes. In addition to their role as mediators of a pain, prostanoids such as PGE_2 mediate various inflammatory responses. Prostanoids have also been implicated in the pathogenesis OA by affecting cartilage matrix integrity and proteoglycan degradation as well as chondrocyte dedifferentiation and apoptosis.[1 6] Cellular effects of prostanoids are mediated through G-protein coupled receptors; many prostaglandin receptor subtypes, particularly DP_1, EP_2, EP_4 and IP,[7] activate adenylate cyclase leading to increased intracellular levels of the multifunctional second messenger cAMP. By activating protein kinase A and transcription factors such as cAMP response element-binding protein, cAMP also regulates the expression of a number of genes.[8] This pathway offers a possible prostanoid-dependent mechanism for the changes in gene expression seen in the present study. In addition, the IPA analysis showed that ibuprofen regulates several other inflammatory pathways which may mediate its effects on chondrocyte transcriptome by prostanoid dependent or independent manner.

In our data, ibuprofen increased the expression of *PPARG* and its coactivator 1 beta (*PPARGC1B*). *PPARG* expression has been shown to be downregulated in OA cartilage,[9] and *PPARG* may affect the pathogenesis of OA by suppressing joint inflammation, downregulating the production of catabolic enzymes and inhibiting

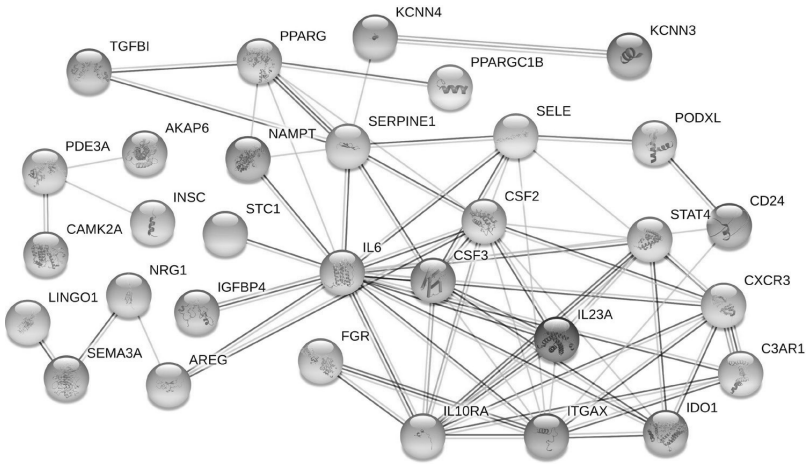

Figure 1 Interactions among the genes that were upregulated or downregulated by ibuprofen with an FC 1.5 or greater (in either direction) in IL-1β-treated cells. Genes with no identified interactions are excluded from the graph. Colours of the edges: green=activation, blue=binding, black=chemical reaction, red=inhibition, violet=catalysis, pink=posttranslational modification, yellow=transcriptional regulation, grey=other interaction.

chondrocyte apoptosis.[10] Induction of some proinflammatory factors such as *CSF2*/GM-CSF by ibuprofen can be regarded as a potentially deleterious effect, as CM-CSF has been shown to promote OA development and pain.[11] To our knowledge, this is the first study linking NSAIDs to GM-CSF production in chondrocytes.

IL6 and *IL23A* as well as *ADAMTS6* (ADAM metallopeptidase with thrombospondin type 1 motif 6) are examples of proinflammatory/catabolic factors that were suppressed by ibuprofen. Ibuprofen downregulated also hyaluronan synthase 1 (*HAS1*) and stanniocalcin-1 (*STC1*) both of which have been shown to be upregulated in inflamed OA joints.[5] These data suggest that ibuprofen can, to some extent, 'normalise' the phenotype of OA tissue under inflammatory conditions. Notably, *IL23A* was the most strongly downregulated gene in our data. The potential local roles of this proinflammatory cytokine in OA cartilage appear relatively understudied, but its serum levels in patients with OA have been found to be higher compared with controls.[12] IL-6 is considered a central proinflammatory mediator in OA.[13] *HAS1* is one of the three principal enzymes participating in the synthesis of hyaluronan, a central extracellular matrix (ECM) component. It may also promote inflammation by producing pericellular, monocyte-attracting hyaluronan coats.[14] *STC1* is a calcium-regulating and phosphate-regulating protein whose effects on cartilage appear to be complex. It may inhibit cartilage development,[15] but its expression in synovial cells has also been linked to slower OA progression.[16]

Integrin signalling was the IPA pathway most strongly activated by ibuprofen. This is interesting, as dysregulated integrin signalling has been implicated in OA pathogenesis.[17] Other significantly upregulated pathways include several linked to inflammation (such as IL-8, NF-κB and MAPK/ERK signalling). Looking at the specific genes included in these pathways and affected by ibuprofen (online supplemental table S6) reveals that these can be mostly considered negative feedback genes rather than the major proinflammatory mediators/effectors of these pathways. Examples include several integrins (*ITGAM*, *ITGAX*, *ITGB2*, *ITGB3* and *ITGB5*) in the IL-8 and NF-κB pathways, growth factors and their receptors (*VEGFA*, *VEGFC*, *HBEGF* and *FGFR3*) in IL-8 signalling as well as anti-inflammatory MAPK phosphatases and PPAR pathway constituents (*DUSP1*, *DUSP2*, *DUSP4*, *PRKAR1A*, *PRKAR1B*, *PRKAR2B* and *PPARG*) in MAPK/ERK signalling.

Intriguingly, PTEN signalling was inhibited by ibuprofen. PTEN is a modulator of phosphoinositide 3-kinase/Akt (PI3K/Akt) signalling with various potential effects including promotion of apoptosis, regulation of cell adhesion and inhibition of cell proliferation. *PTEN* is upregulated in OA chondrocytes, where it inhibits the production of ECM components,[18] and interventions that inhibit PTEN slow the development of osteoarthritic changes in cartilage.[19] To our knowledge, PTEN has not previously been linked to NSAIDs in cartilage.

Previous studies have investigated the effects of NSAIDs and COX-2 selective inhibitors on cartilage/synovial explants.[6 20] Both prostaglandin-mediated and prostaglandin-independent effects have been observed; these include, for example, inhibition of chondrocyte apoptosis, reduction of nitric oxide synthesis as well as reduced production of catabolic MMPs on IL-1β stimulation.[1] Our study expands these results by investigating the whole transcriptome of ibuprofen-treated OA chondrocytes and provides a starting point for future studies.

In conclusion, ibuprofen alone had no significant effects on gene expression in chondrocytes supporting

cartilage safety of COX inhibitors in the treatment of OA pain. When used in a setting of joint inflammation, ibuprofen seems to shift chondrocyte transcriptome towards an anti-inflammatory phenotype.

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