NTNU – Trondheim
Norwegian University of
Science and Technology

Effects of Toll-like Receptor Agonists and Tumor Necrosis Factor-α in the SW982 Cell Model for Synovitis

Trine Skuland

Biotechnology (5 year)
Submission date: May 2013
Supervisor: Berit Johansen, IBI
Co-supervisor: Astrid J. Feuerherm, IBI

Norwegian University of Science and Technology
Department of Biology

Abstract

Rheumatoid arthritis (RA) is a chronic, autoimmune joint disease characterized by a massive infiltration of immune cells and synovial lining hyperplasia. This ultimately leads to synovitis, i.e. inflammation of the synovial membrane, and excessive bone loss. Certain bone remodeling components are known to play a key role in the RA pathogenesis: receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and dickkopf homolog 1 (DKK1). RANKL is a ligand essential to differentiation of the bone-degrading osteoclasts, while OPG can hinder this by competitive binding of RANKL. DKK1 inhibits activation of the bone-forming osteoblasts. In addition, toll-like receptors (TLRs) are believed to be central in RA. TLRs bind pathogen associated molecular patterns (PAMPs), but also damage associated molecular patterns (DAMPs) found on molecules present in inflamed joints. TLR ligand binding activates production of pro-inflammatory mediators.

The exact events and pathways involved in RA are not fully understood. By using the synovial fibroblast (SF) cell line, SW982, as a model for synovitis, this master's thesis aims to clarify certain signaling events. More specifically, the potential involvement of phospholipase A_2 (PLA_2) and cyclooxygenase (COX) in expression of bone remodeling genes, in response to TLR agonists and tumor necrosis factor (TNF)-α, was investigated. A known downstream effect of TLR activation is production of the pro-inflammatory cytokine TNF-α. Consequently, TNF-α's effect on the TLR expression was also studied.

Gene expression analysis, by quantitative polymerase chain reaction (qPCR), revealed that the SW982 cells express OPG and DKK1, and probably RANKL. In addition, the cells express TLR1-6, and possibly TLR7 too. It was shown, for the first time, that a TLR1/2 agonist (Pam3CSK4), a TLR2/6 agonist (FSL-1), and a TLR3 agonist (Poly(I:C)), increase the OPG and DKK1 gene expression in SW982 cells. The OPG mRNA increase was also detected at protein level by performing enzyme-linked immunosorbent assay (ELISA). Furthermore, activation of TLR1/2 and TLR2/6 was found to strongly induce IL-6 and COX-2 gene expression. It was discovered that $cPLA_2$ and COX are involved in TLR1/2-mediated induction of DKK1, IL-6 and COX-2 gene expression, and possibly OPG expression. For the TLR2/6-mediated expression of these genes, the involvement of $cPLA_2$ and COX is not as pronounced as for TLR1/2, but still likely. Altogether, these results indicate that the prostaglandin (PG) pathway is triggered upon TLR activation. Besides affecting the mentioned genes, this will lead to increased production of PGs. Prominent PG production is often observed in inflammatory conditions like RA.

The activity of PLA_2s in response to TLR agonists was studied by radioactivity assays. Activation of TLR1/2, TLR2/6 and TLR3 was found to increase the release of the inflammatory intermediate arachidonic acid (AA) – the first precursor of PG synthesis. Further assays suggested that the GIVA $cPLA_2$ is the main PLA_2 responsible for the increase in AA release, with possible involvement of Ca^{2+}-independent PLA_2 ($iPLA_2$).

Upon investigation of TNF-α's effects, it was found that the SW982 cells increase their DKK1, TLR2 and TLR3 gene expression. The no-response results for RANKL, OPG, TLR1, and TLR4-7, and the no-response results regarding $cPLA_2$'s involvement, were inconclusive due to non-optimal TNF-α stimulation.

In conclusion, even though the bone-protective OPG is up-regulated by TLR activation, this activation may have more negative effects in the context of RA: DKK1, COX-2, IL-6 and AA release are increased, and these are all contributors of joint destruction in RA. In addition, OPG may also exert a negative effect. The protein can prevent apoptosis of SFs and thereby contribute to synovial hyperplasia. Because $cPLA_2$ seems to be involved in the increase of the mentioned components, the enzyme may be an attractive target for reducing inflammatory responses set off by TLRs.

Sammendrag

Revmatoid artritt (RA) er en kronisk, autoimmun leddsykdom karakterisert ved massiv immuncelle-infiltrasjon og hyperplasi av synovialhinnen. Dette fører til synovitt, dvs. betennelse i synovialmembranen, og bentap. Det er kjent at visse benutformingskomponenter er viktige i RA-patogenesen, nemlig reseptoraktivator av nukleær faktor-κB-ligand (RANKL), osteoprotegerin (OPG) og dickkopf-homolog 1 (DKK1). RANKL er nødvendig for differensiering av bennedbrytende osteoklaster, mens OPG kan forhindre dette ved konkurrerende binding av RANKL. DKK1 hemmer aktivering av bendannende osteoblaster. I tillegg er Toll-lignende reseptorer (TLRer) også sentrale i RA. TLRer binder til patogen-assosierte molekylære mønstre (PAMPer), men kan også binde til skade-assosierte molekylære mønstre (DAMPer) som finnes på visse molekyler i betente ledd. TLR-ligandbinding aktiverer produksjon av proinflammatoriske faktorer.

De eksakte hendelsene og signalsporene involvert i RA er ikke fullsteding kjent. Ved å bruke synovial fibroblast (SF) -cellelinjen, SW982, som modell for synovitt har denne masteroppgaven som mål å avklare enkelte signaleringshendelser. Det ble undersøkt om fosfolipase A_2 (PLA_2) og syklooksygenase (COX) er medansvarlige for uttrykk av benutformingsgener ved respons på TLR-agonister eller tumornekrosefaktor (TNF) -α. Produksjon av det proinflammatoriske cytokinet TNF-α er en kjent nedstrøms konsekvens av TLR-aktivering. Derfor ble det også undersøkt om TNF-α hadde innvirkning på TLR-uttrykk.

Genuttrykksanalyse, gjort ved hjelp av kvantitativ polymerase kjedereaksjon (qPCR), viste at SW982-celler uttrykker OPG og DKK1, og sannsynligvis RANKL. I tillegg uttrykker cellene TLR1-6, og trolig også TLR7. For første gang ble det vist at en TLR1/2-agonist (Pam3CSK4), en TLR2/6-agonist (FSL-1), og en TLR3-agonist (Poly(I:C)) resulterer i økt OPG- og DKK1-genuttrykk i SW982-celler. Økningen av OPG på mRNA-nivå ble også funnet på protein-nivå ved å utføre enzymkoblet immunabsorbsjonsanalyse (ELISA). Videre ble det påvist at aktivering av TLR1/2 og TLR2/6 induserer IL-6- og COX-2-genuttrykk i stor grad. Det ble også funnet ut at cPLA2 og COX er involvert i TLR1/2-mediert induksjon av DKK1-, IL-6- og COX-2-genuttrykk, og muligens OPG-uttrykk. For TLR2/6-mediert uttrykk av disse genene er ikke medvirkningen til cPLA2 og COX like tydelig, men likevel sannsynlig. Disse resultatene indikerer at prostaglandin (PG)-signalsporet aktiveres ved TLR-signalisering. I tillegg til å påvirke de nevnte genene vil dette føre til økt produksjon av PGer, noe som ofte forekommer ved inflammatoriske sykdommer som RA.

Aktiviteten til PLA_2-enzymer ved respons på TLR-agonister ble undersøkt ved hjelp av radioaktivitetsanalyser. TLR1/2-, TLR2/6- og TLR3-aktivering førte til økt frigjøring av den inflammatoriske komponenten arakidonsyre (AA) - den første forløperen i syntese av PGer. Ytterligere analyser antydet at GIVA $cPLA_2$ er hovedansvarlig for den økte AA-frigjøringen, med mulig medvirkning av Ca^{2+}-uavhengig PLA_2 ($iPLA_2$).

Det ble funnet at SW982-cellene øker sitt DKK1-, TLR2- og TLR3-genuttrykk etter TNF-α-stimulering. Det at ingen induksjon ble detektert for RANKL, OPG, TLR1, og TLR4-7, og likeså den manglende responsen i forhold til $cPLA_2$s medvirkning, gjenspeiler muligens ikke realiteten. Dette fordi TNF-α-stimuleringen i disse forsøkene ikke var optimal.

Selv om ben-beskyttende OPG oppreguleres ved TLR-aktivering, ser det ut til at aktiveringen vil kunne ha flere negative konsekvenser i en RA-sammenheng: Nivåene av DKK1, COX-2, IL-6 og AA-frigjøring er også forhøyet, og disse bidrar alle til leddødeleggelse i RA. OPG kan i tillegg ha negativ virkning. Proteinet kan nemlig forhindre apoptose av SFer og dermed bidra til synovial hyperplasi. Ettersom $cPLA_2$ synes å være involvert i økningen av de nevnte komponentene, kan dette enzymet være et attraktivt mål for å redusere inflammatoriske responser som utløses av TLRer.

Preface

This master's thesis was performed as a finalizing part of the master programme (5 years) in biotechnology (MBIOT5) at the Norwegian University of Science and Technology (NTNU). The research was executed in the PLA_2 group at Department of Biology, and the working period was from August 2011 to May 2013. The main supervisor was professor Berit Johansen, and co-supervisors were senior researcher Astrid Jullumstrø Feuerherm and PhD candidate Randi Magnus Sommerfelt.

First and foremost, I would like to express my gratitude to Astrid. Thank you so much for being so positive and inspiring in teaching me laboratory techniques and how to think like a biological scientist. You made it easier for me to be a calf! Like you said: "Everyone started out as calves!" Moreover, I am very grateful to you for always finding time to answer my questions and to encourage me whenever I was stressed out. I also truly appreciate Randi's helpful tips to both laboratory work and thesis writing. Similarly, thanks a lot to Berit for excellent input when needed! In addition, I would like to thank the entire PLA_2 group for providing a good working environment and for their willingness to help. Particularly to Thuy: Thank you so much for your skilled qPCR help!

Last but not least, I would like to mention my beautiful family and friends. My parents and my sisters are all hard workers, and I strive to be like them. Thank you for being great role models and for always believing in me! The biggest thanks I probably owe to Fredrik, my dear boyfriend, who has been my patient roommate for this entire period. Thank you for all your computer help and all other help related to my thesis work, and thanks a lot for your kind support! What should I have done without you? Finally, thanks to all my good friends who have been there for me whenever I needed to relax or let out my frustrations.

Trondheim, May 2013

Trine Skuland

List of abbreviations

AA	Arachidonic acid
Anti-CP	Autoantibody to citrullinated peptides
Anti-RA33	Autoantibody to RA antigen of 33 kDa
AP	Activating protein
APC	Antigen-presenting cell (except in figure 1.2.2; adenomatous polyposis coli gene product)
APRIL	A proliferation inducing ligand
ATCC	American Type Culture Collection
AVX002	$cPLA_2$ inhibitor: 1-octadeca-3,6,9,12,15-pentaenylsulfanyl-propan-2-one
BAFF	B cell activating factor
BEL	Bromoenol lactone, $iPLA_2$ inhibitor: 6E-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one
Blys	B lymphocyte stimulator
C’	Complement
CAM	Cell adhesion molecule
CAY10502	$cPLA_2$ inhibitor: 1-[3-[4-(decyloxy)phenoxy]-2-oxopropyl]-1H-indole-3,5-dicarboxylic acid, 3-methyl ester
CAY10590	$sPLA_2$ inhibitor: 4-[(1-oxo-7-phenylheptyl)amino]-(4R)-octanoic acid
CCL	CC-chemokine ligand
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CIA	Collagen-induced arthritis
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2

C_q	Quantification cycle (also known as threshold cycle)
CR	Complement receptor
C_T	Threshold cycle
CXCL	CXC-chemokine ligand (CXC: a chemokine subgroup where conserved cysteins are separated by some other amino acid, X)
DAG	Diacylglycerol
DAMP	Damage associated molecular pattern
dH_2O	Distilled water
DKK1	Dickkopf homolog 1
DMARD	Disease-modifying anti-rheumatic drug
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Dsh	Disheveled
2D	Two-dimensional
3D	Three-dimensional
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EP	Prostaglandin E_2 receptor
ERK	Extracellular-signal regulated kinase
fBSA	Fatty acid free bovine serum albumin
FBS	Fetal bovine serum
FcR	Fc receptor (receptor for the Fc portion of IgG)
FSL-1	Synthetic diacylated lipoprotein: (S,R)-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe

gp96	Endoplasmic reticulum stress response protein
GSK3	Glycogen synthase kinase-3
HRP	Horseradish peroxidase
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
HSP	Heat shock protein
IC	Immune complex
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IKK	I-kappa B kinase
IκBα	Inhibitor of NF-κB
IL	Interleukin
Indomethacin	COX inhibitor: 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid
InhibX	$cPLA_2$ inhibitor (name and structure not published)
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor-3
JNK	c-jun N-terminal kinase
$LpPLA_2$	Lipoprotein-associated phospholipase A_2
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
LT	Lymphotoxin
MAFP	Methyl arachidonyl fluorophosphonate, $iPLA_2$ and $cPLA_2$ inhibitor: 5Z, 8Z, 11Z, 14Z-eicosatetraenyl-phosphonofluoridic acid, methyl ester

MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MD	Myeloid differentiation protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MyD88	Myeloid differentiation primary-response protein-88
NF-κB	Nuclear factor-κB
NLR	Nod-like receptor
NSAID	Non-steroidal anti-inflammatory drug
OA	Oleic acid
OPG	Osteoprotegerin
PAMP	Pathogen associated molecular pattern
PAP	Phosphatidic acid phosphohydrolase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PGN	Peptidoglycan
PI3	Phosphatidylinositol 3-kinase
λ/ιPKC	λ/ι protein kinase C
PLA_2	Phospholipase A_2
PIC	Poly(I:C), synthetic analog of double-stranded RNA: Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor

P3C	Pam3CSK4, synthetic triacylated lipoprotein: N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl- [S]-lysyl-[S]-lysyl-[S]-lysine
4-PL	Four parameter logistic
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RF	Rheumatoid factor
RIP1	Receptor interacting protein 1
PKC	Protein kinase C
RNA	Ribonucleic acid
SCID	Severe combined immunodeficient
SD	Standard deviation
SEM	Standard error of mean
SF	Synovial fibroblast (also known as fibroblast-like synoviocyte)
SF-DMEM	Serum free DMEM (only L-glutamine and gentamicin added, no FBS)
SNP	Single-nucleotide polymorphism
$sPLA_2$	Secreted phospholipase A_2
TACI	Transmembrane activator and calcium modulator cyclophilin ligand interactor
TAE	Tris acetate-EDTA
TBK	TANK-binding kinase
TCR	T cell receptor
TGF	Transforming growth factor
Th1	T helper 1 cell
Th17 cells	IL-17 producing T cells

TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-α receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cell
TRIF	TIR domain-containing adaptor-inducing IFN- β
TX	Thromboxane
Varespladib	$sPLA_2$ inhibitor: 2-(3-(2-amino-2-oxoacetyl)-1-benzyl-2-ethyl-1H-indol-4-yloxy) acetic acid
WNT	Wingless

Table of Contents

1. INTRODUCTION

1.1 The pathogenesis of rheumatoid arthritis

About 0.5-1% of the population in the industrialised world is affected with rheumatoid arthritis (RA), which is the most common chronic joint disease [1]. This is an autoimmune disease, meaning that the immune system is activated by self-molecules. RA is not caused by one specific event, but rather by a complex interplay of certain genotypes, environmental triggers, and chance. For example, several RA risk alleles have been identified, and smoking and infectious agents have been linked to the disease. Moreover, women have a greater risk of developing RA than men [2]. The disease can appear at any age, but most commonly affects people in their forties to sixties [3].

The most common joints attacked in RA patients are those of hands, feet and knees [3]. The joints acquire a characteristic synovitis, which is inflammation of the synovial membrane. The synovitis has a special tendency to invade cartilage and articular bone – the formation of a so-called pannus. The pannus consists of immune cells, blood vessels and fibrous cells [4]. The pannus invades both cartilage and bone, comparable with the behaviour of an invasive tumour [5]. Joint pain, stiffness, and swelling are some of the perceptible symptoms of RA. The disease causes disability and without proper treatment the outcome can be premature death [1, 4]. The saying "misfortunes never come singly" also applies to RA. Several co-morbid conditions are identified. Some of the major ones are cardiovascular disease, infection, lymphoproliferative malignancy, gastrointestinal disease, and osteoporosis [6].

1.1.1 The innate immune system signals to the adaptive immune system

The sequence of the disease events of RA is not fully elucidated. However, a natural place to start is with the abundance of immune cells that enter the synovium when a joint is affected by RA, as shown in figure 1.1.1 [1]. The entering process is called extravasation. It is mediated by special cell adhesion molecules (CAMs) expressed by the activated vascular endothelium cells and the corresponding receptors for these CAMs on the immune cells. The CAMs are expressed in response to specific cytokines produced in inflammatory responses [7]. Cells of the innate immune system, such as dendritic cells and macrophages, express pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are examples of PRRs, which recognize specific pathogen associated molecular patterns (PAMPs). Recently, it has been discovered that the TLRs also can detect endogenous molecules; so-called damage associated molecular patterns (DAMPs) [8]. A more detailed description of the TLRs and their involvement in RA can be found in section 1.3.

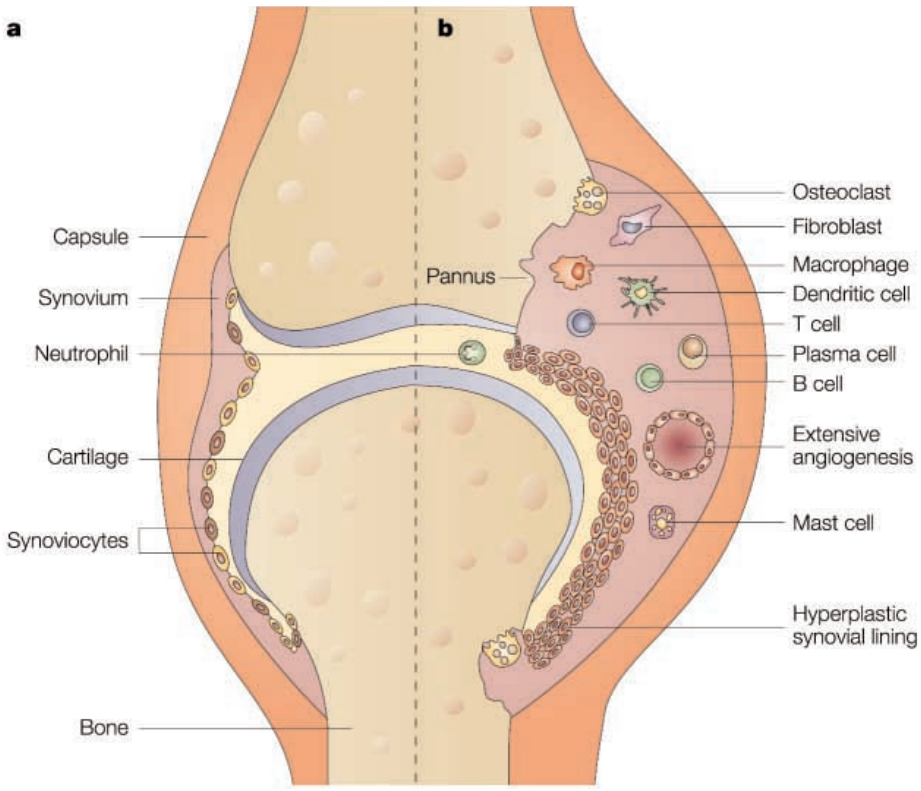

Figure 1.1.1 The differences between a) a normal joint and b) a RA affected joint [3].

A cell becomes activated when TLR bind its ligand, and the cell will then send the message further on to the adaptive immune system. Some of the immune cells bearing TLRs are professional antigen-presenting cells (APCs), such as the dendritic cells and macrophages. When activated, these cells will take up and present RA antigens, via their membrane bound class II major histocompatibility complex (MHC) molecules, to T cells. The interaction of the T cell receptor with the presented antigen is the first requirement for T cell activation. The second requirement is the binding of a co-stimulatory ligand, either cluster of differentiation (CD) 80 or CD86, on an APC to the CD28 receptor on the T cell. This will fully activate the T cell to start producing various cytokines. Secretion of cytokines like interleukin (IL)-2 and interferon (IFN)-γ causes the activated T cell to proliferate and differentiate into memory or effector T cell populations [1]. A subset of T cells, called regulatory T cells (Treg), function as a control of T cell activation by suppression. In RA, it is found that these regulatory cells have reduced function [1, 4].

The effector T cells in RA synovium are mostly of the T helper 1 (Th1) subset. The Th1 cells induce activation of macrophages, B cells, fibroblasts, and osteoclasts. B cells express membrane-bound immunoglobulins (Igs), which are molecules that bind antigens. Antigen binding by the Igs leads to increased expression of class II MHC molecules with bound antigen, and the B cells thus act as APCs to the Th1 cells. At the same time, the CD40 receptor on the B cells interacts with the CD40 ligand on the Th1s leading to activation of the B cells. Because the B cell also function as an APC to activate T cells, this can initiate a vicious cycle that keeps the autoimmune response going. When activated, B cells will start their differentiation into antibody

secreting plasma cells. The secretion includes autoantibodies, such as rheumatoid factor (RF; autoantibody to IgG), autoantibody to RA antigen of 33 kDa (anti-RA33), and autoantibody to endogenous citrullinated peptides (anti-CP) like vimentin or fibrinogen [1]. RFs are reactive with the Fc region of the IgG and can form immune complexes by binding to normal circulating IgGs. These complexes can activate the complement system leading to an inflammatory response called type III hypersensitivity, which is mediated by massive infiltration of neutrophils. In addition, the autoantibodies can bind to Fc receptors (FcRs) on e.g. macrophages, causing increased production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) [1, 7]. The events of RA pathogenesis are graphically summarized in figure 1.1.2.

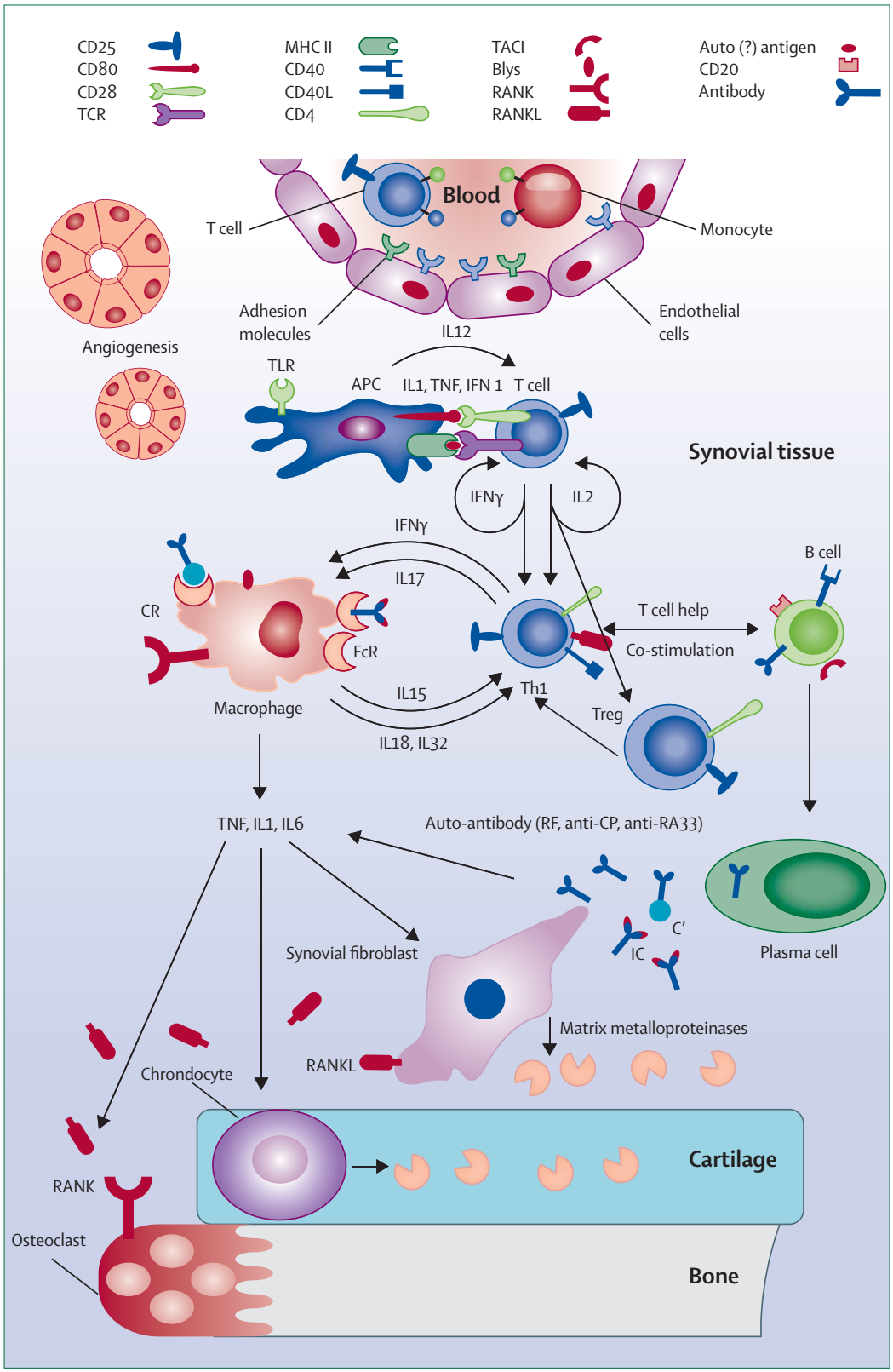

Figure 1.1.2 An overview of the events in RA pathogenesis [1]. anti-CP, autoantibody to endogenous citrullinated peptides; anti-RA33, autoantibody to RA antigen of 33 kDa; APC, antigen-presenting cell; Blys, B lymphocyte stimulator; C', complement; CD, cluster of differentiation; CR, complement receptor; FcR, receptor for the Fc portion of IgG; IC, immune complex; IFN, interferon; IL, interleukin; MHC II, major histocompatibility complex class II; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; RF, rheumatoid factor; TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; TCR, T cell receptor; Th1, T helper 1 cell; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cell

Except from the antibodies being self-reactive, all the processes described above take place in a normal inflammatory response initiated to destroy and eliminate invading pathogens. This response will eventually come to an end when the mission is accomplished. However, in RA the immune response persists and becomes chronic [9].

1.1.2 Synovial fibroblasts are key players in RA

Beside the already mentioned immunocompetent cells, synovial fibroblasts (SFs) are also known to accumulate in the synovium. SFs are often accused of being the key effector cells in RA [1, 5]. In a normal joint, the synovial membrane is about 1-2 cells deep and consists of two synoviocyte types; type A and type B. Type A are macrophage-like synovial cells and type B are the SFs. In a RA synovial membrane, however, the amount of both cell types increases significantly, as shown in figure 1.1.1. The lining can expand to a depth of 10-20 cells. What triggers this expansion of the synoviocyte population is not fully elucidated. It is likely to be caused by an imbalance between cell proliferation, survival, and death. The environment in a RA synovium seems to prevent apoptosis of SFs and favor their survival [5].

The expanded macrophage-like cell population in the synovium becomes highly activated. This leads to production of an abundance of cytokines, chemokines, and growth factors, which further activate the SFs. Activated SFs also secrete a number of inflammatory mediators, such as cytokines (especially IL-6), chemokines, prostanoids, and matrix metalloproteinases (MMPs). These compounds act in a paracrine and/or autocrine fashion, and in this way they perpetuate the synovitis and attract new immune cells [5, 10]. In addition, the cytokines also stimulate the chondrocytes, which subsequently secrete MMPs. The MMP enzymes contribute to destruction of cartilage [11].

Since SFs in RA synovium express high levels of class II MHC molecules, it has been reasoned that these cells also act as APCs for the T cells. However, studies have shown that the SFs are not as efficient in taking up and presenting antigens as the professional APCs. Still, it is of interest to evaluate their role in autoimmunity as non-professional APCs [10].

RA originates in a few joints, but it is progressive and can spread to several joints. Studies have shown that the cells responsible for this spreading are the SFs. Using severe combined immunodeficient (SCID) mouse models it was demonstrated that human RA SFs could attach to and invade distant cartilage of unaffected joints after migrating through the bloodstream [12].

1.1.3 Bone destruction by osteoclasts

A cell type called osteoclasts, which differentiate within the synovial membrane from monocyte and macrophage precursors, causes the bone erosion observed within joints of RA patients [1]. The osteoclasts are an essential part of the normal bone metabolism, and they are the primary bone resorbing cells with their two central resorbing machineries. These two are the proton/protein pump that acidifies the milieu, allowing calcium to get solubilized, and the matrix degrading enzymes like MMPs and cathepsins. Because of these inherent properties, the osteoclasts can create resorption pits in bone. Normally, the bone synthesizing osteoblasts will fill these pits again to maintain bone homeostasis [13]. This balance is disrupted in RA and bone degradation is favored, as will be described in further detail in section 1.2.

The main requirement for the osteoclast precursors to differentiate into mature osteoclasts and to be activated, is that their membrane-bound receptor activator of nuclear factor-κB (RANK) must bind the receptor activator of nuclear factor-κB ligand (RANKL) [14]. As shown in figure 1.1.2, the cells that stand out by their RANKL expression in RA are the activated T cells and SFs [15]. Macrophage colony-stimulating factor (M-CSF) is also necessary for osteoclast differentiation. Synovial mesenchymal cells and T cells secrete M-CSF. Like RANKL, M-CSF binds to its receptor on the osteoclast precursors [4].

1.1.4 Cytokines

As they do in all inflammatory responses, cytokines play a major role in RA [1]. The cytokines are a part of a complex regulatory network. By binding to their receptors on different cells acting in series and in parallel, they contribute to autoimmunity, chronic inflammation and tissue destruction in RA [4, 16].

The cytokines are pleiotropic, meaning that one single cytokine can have several effects. They are also redundant. That is, none of the mediated events depend on only one cytokine [16]. All the accumulating cells hereto mentioned, secrete various cytokines and are affected by them. Only a few selected cytokines will be described here to demonstrate their importance. E.g., IL-15 is a major cytokine growth factor for synovium T cells. The Th1 subset is an important effector in RA, as previously mentioned. A more recent model suggests that IL-17 producing T cells (Th17 cells) also are crucial effectors. The differentiation of Th1 and/or Th17 cells are promoted by several cytokines derived from macrophages and SFs, such as IL-1β, IL-6, IL-7, IL-12, IL-15, IL-18, IL-23p19, and transforming growth factor (TGF) -β. Dendritic cells also produce some of these cytokines, and are therefore thought to influence the T-cell differentiation [4].

B cells contribute to RA pathogenesis by producing cytokines like IL-6, IL-10 and lymphotoxin (LT) -β. These cytokines participate in events such as activation of follicular dendritic cells and in lymphoid regeneration [4].

The most important cytokine producers in the synovium are the macrophages, which are derived from monocytes. TLR ligand binding is likely to be responsible for the activation of these cells and their subsequent cytokine production [4]. FcR binding of immune complexes can also stimulate monocytes to produce cytokines. Macrophages release a range of different cytokines. The pro-inflammatory cytokine TNF is one of them, and is considered of primary importance in RA. Studies have shown that TNF inhibition suppresses arthritis in different models, and TNF overexpression conduces to erosive, inflammatory arthritis. Among the effects of TNF is induction of some of the major pathological processes in RA, such as leukocyte and endothelial-cell activation, SF activation and survival, pain-receptor sensitization and development of new blood vessels (angiogenesis) [4]. Consequently, TNF was considered an attractive therapeutic target and the development of TNF inhibitors revolutionized the treatment of RA. Although the inhibitors cannot cure the disease, they make it more manageable for a majority of patients [17]. IL-1 and IL-6 are also important pro-inflammatory cytokines derived from macrophages. Both TNF and IL-1 can induce IL-6 production. IL-6 contributes to RA through its induction of B cell antibody production, T cell, macrophage, and osteoclast activation. It also has an important role in activation of the acute-phase response in the liver [1]. On a similar level to TNF

inhibition, blockade of the IL-6 receptor is quite effective in reducing bone degradation in RA. Other pro-inflammatory cytokines, such as IL-1, IL-15 and IL-17, are also subjected as therapeutic targets [18].

In summary, pro-inflammatory cytokines are central in RA. A direct example is that TNF-α, IL-1, IL-6 and IL-17 affect bone erosion by increasing the expression of RANKL [13]. In addition, TNF and IL-7 promotes M-CSF production, which, together with RANKL, is required for osteoclast differentiation and activation. IL-1β also regulates the expression of RANK, and contributes to cartilage degradation through matrix synthesis inhibition and induction of matrix-degrading enzymes. IL-17 induces both TNF and IL-1β expression in SFs, perpetuating the inflammation. Moreover, cytokines are likely to play a part in regulation of bone formation. TNF has been shown to inhibit differentiation and function of osteoblasts, and to up-regulate secretion of dickkopf homolog 1 (DKK1) [4]. The DKK1 protein inhibits bone formation, and its involvement in RA is further explained in section 1.2.

A simplified overview of the cytokine-mediated events in the RA synovium is given in figure 1.1.3. All the processes presented in the figure are not explained in this introductory chapter, due to restriction of what is considered relevant for this master's thesis. The aspects that are elaborated in this introduction are highlighted in red.

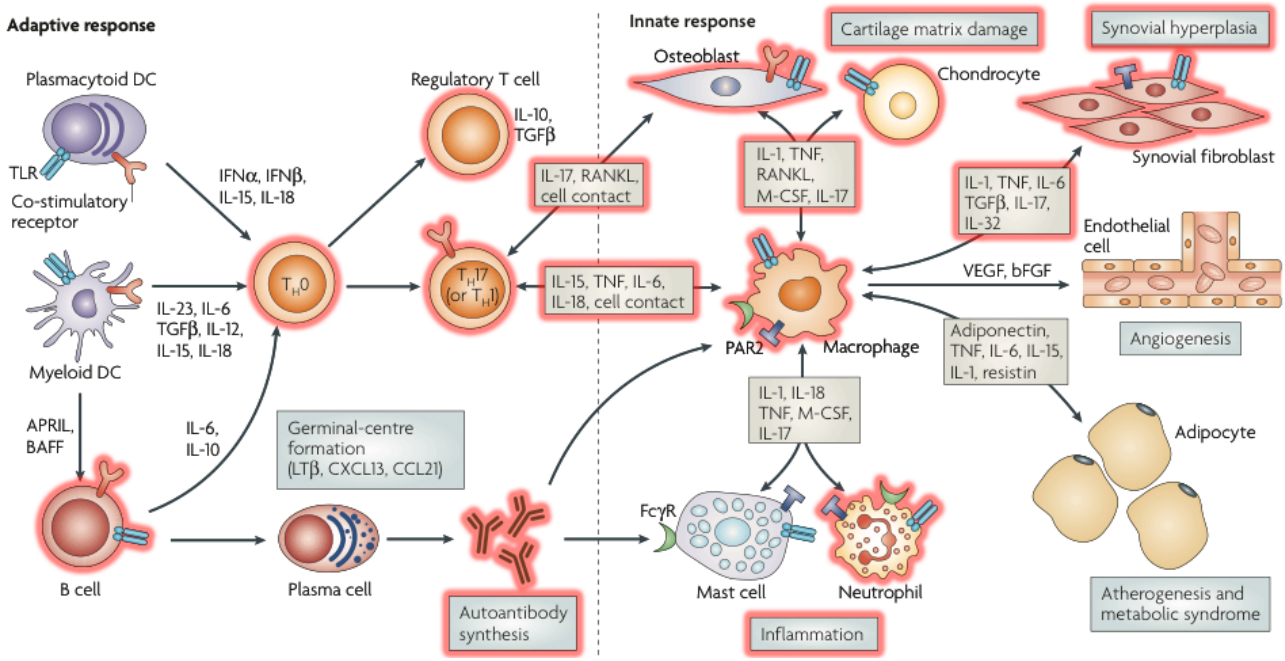

Figure 1.1.3 A simplified overview of the cytokine-mediated events in the RA synovium [4]. The red highlighting indicates the aspects that are described in further detail in this introductory chapter. APRIL, a proliferation inducing ligand; BAFF, B cell activating factor; CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; DC, dendritic cell; FcR, receptor for the Fc portion of IgG; IFN, interferon; IL, interleukin; LT, lymphotoxin; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand; TGF, transforming growth factor; Th, T helper cell; Th17, IL-17 producing T cells; TLR, Toll-like receptor; TNF, tumor necrosis factor

Some cytokines can also have anti-inflammatory effects. In RA, these and other endogenous anti-inflammatory compounds, like soluble cytokine receptors, enzyme inhibitors and receptor antagonists, have insufficient activity to counteract the inflammatory response [3].

1.1.5 Other important molecules

There are other important molecules involved in RA, such as chemokines and cell adhesion molecules. Both bind to specific receptors and in this way carry out functions like cell interactions, migration, and chemoattraction of cells [1]. Macrophages and SFs in the synovial lining produce chemokines and other small chemoattractant molecules that recruit immune cells, which are essential for synovitis development. Most chemokines from the synoviocytes makes immune cells migrate into the joint, but some also facilitate angiogenesis. For example IL-8 is a chemokine that act both as a potent neutrophil attractant and a stimulator of blood vessel formation [5].

1.2 Key proteins involved in bone remodeling

Bone remodeling is a process that takes place throughout life. It is essential for adapting bone strength, repairing damage, and maintaining blood calcium levels. As stated earlier, osteoclasts are the cells responsible for the formation of resorption pits. The osteoblasts follow behind, filling in these pits again with new bone matrix, as shown in figure 1.2.1. Normally there is a balance between the functions of these two cell types, but in RA this balance is disrupted. The inflammatory bone loss observed in RA is due to increased activity of osteoclasts and decreased activity of osteoblasts [19].

1.2.1 RANKL and OPG: inducing or preventing osteoclast activation

The osteoblasts express a range of characteristic cell membrane molecules. Among these are RANKL. RANKL is a cytokine of the TNF superfamily, and exists in a soluble form too. As previously mentioned, RANKL binds to RANK on osteoclasts. This interaction is essential to osteoclast differentiation and activation. In RA affected joints, RANKL is also expressed by SFs and T cells [14, 19].

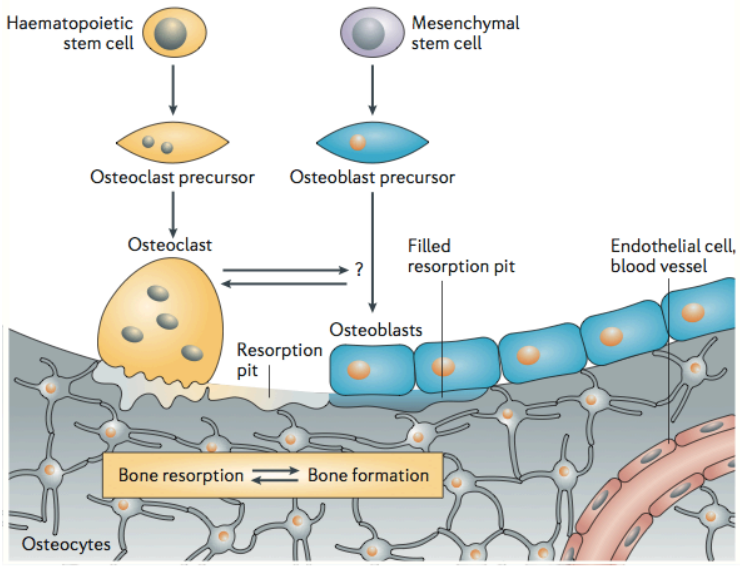

Figure 1.2.1 Bone homeostasis is maintained by bone remodeling carried out by osteoclasts and osteoblasts [19].

Osteoblasts also produce and secrete a protein called osteoprotegerin (OPG). OPG is a part of the TNF superfamily of receptors, and functions as a soluble decoy receptor for RANKL. By competitively binding RANKL, OPG prevents osteoclast activation and consequently bone resorption [14, 19]. In RA synovium, other cells also express OPG, predominantly macrophages and endothelial cells, but there is still an excess of RANKL. This imbalance promotes bone loss [20]. In a study done by Feuerherm and co-workers, both sera and synovial fluids from RA patients were found to have elevated levels of OPG protein [21], whereas other reports show that OPG protein levels are down-regulated in RA synovial fluids [22]. These different discoveries may reflect the fact that it is the RANKL/OPG ratio that controls bone homeostasis, not RANKL or OPG individually. This is supported by the finding that RA patients with an active disease have up-regulated expression of both RANKL mRNA and protein in the synovial tissue, but reduced levels of OPG protein [23]. Such a microenvironment favors osteoclast differentiation and activation [24]. Moreover, it has been found that RA tissue express the RANKL protein in all the cells throughout the synovial lining, but it is considerably up-regulated at the pannus-bone interface. In contrast, OPG protein expression was minimal at this interface. However, cells of the synovial membrane not in the vicinity of bone had a higher OPG protein expression [25]. This points to the likelihood of RANKL/OPG ratio at the pannus-bone interface being the critical factor of the bone erosion in RA [24].

1.2.2 DKK1: inhibiting osteoblast differentiation

Among the systems necessary to activate the osteoblasts is the Wingless (WNT)-Frizzled-β-catenin signaling pathway [24]. WNTs are a group of highly conserved glycoproteins, especially secreted in areas of mixed cell populations. Frizzled, together with lipoprotein receptor-related proteins (LRPs), form the receptor complex that the WNTs bind to. Upon binding, Frizzled passes on the signal to β-catenin, which then acts as a transcription factor. It enters the nucleus and, together with co-activators, it activates transcription of genes involved in osteoblast differentiation. [19, 24]. This pathway is demonstrated in figure 1.2.2. A consequence of ongoing WNT signaling is increased OPG production, along with decreased RANKL expression [26]. In absence of WNT signaling, a complex of proteins degrades β-catenin and it is no longer available to activate gene transcription [24].

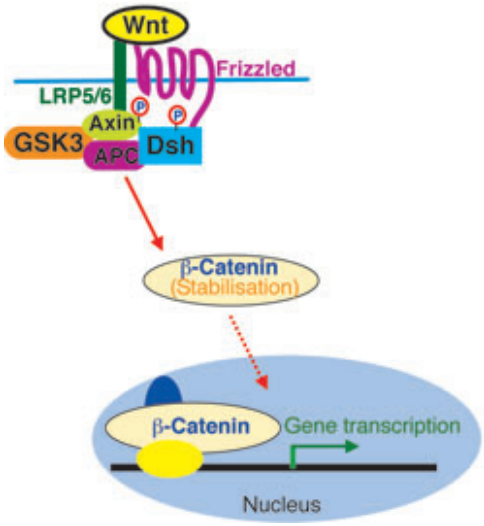

Figure 1.2.2 Signaling of WNT pathway [24]. APC, adenomatous polyposis coli gene product; Dsh, disheveled; GSK3, glycogen synthase kinase-3; LRP, lipoprotein receptor-related protein; Wnt, Wingless

Several endogenous molecules act as inhibitors of the WNT pathway, hence restricting osteoblast function. One of them is the previously introduced DKK1. This protein is secreted in increased amounts by cytokine-activated cells, but can in general be found in normal tissues like the spleen and the skin. The DKK1 works by binding to LRP4, LRP5 or LRP6 with assistance from a co-receptor. This complex is subsequently endocytosed, and the LRP is no longer accessible to WNT binding on the cell surface [19]. It has been found that DKK1 levels are increased in arthritic synovial tissues and in blood serum of RA patients [26]. The SFs was especially prominent in expressing DKK1 in the synovium. Moreover, TNF induces increased DKK1 expression in both mouse and human arthritic SFs. Consequently, the up-regulated DKK1 levels lead to increased inhibition of WNT signaling and reduced osteoblast function. As a result the balance between bone resorption and deposition ceases in RA, and it tips towards resorption as shown in figure 1.2.3 [24, 27].

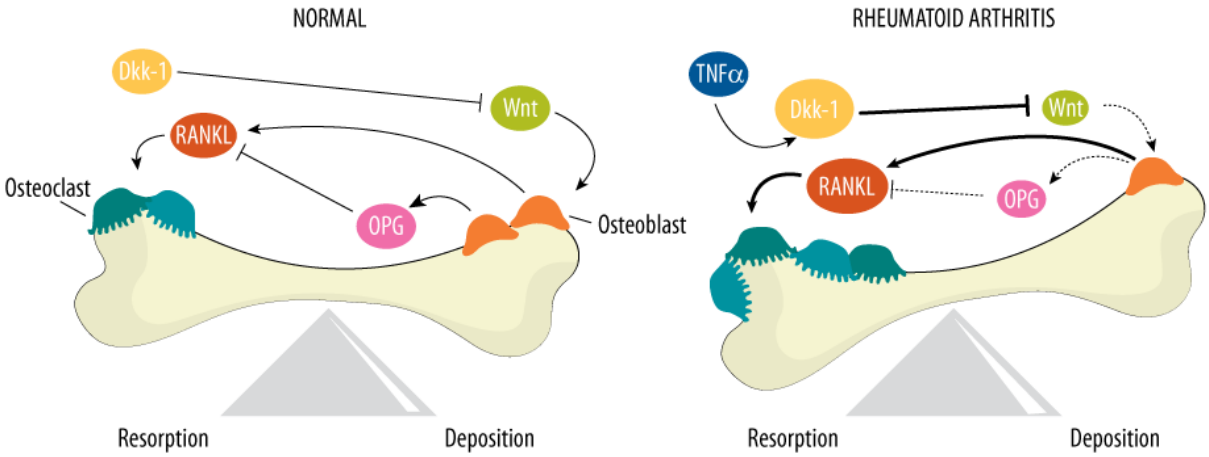

Figure 1.2.3 To the left, the normal balance that exists between bone resorption and deposition is shown. In RA, however, increased DKK1 levels due to TNF expression disrupt this balance, and consequently the weight tips toward resorption, as shown to the right [27]. Dkk-1; dickkopf homolog 1; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TNF, tumor necrosis factor; Wnt, wingless

1.3 Toll-like receptors and their role in rheumatoid arthritis

As previously stated, the TLRs are a part of the innate immune system. Cells like fibroblasts, myeloid cells, epithelial and endothelial cells, express these receptors [8]. TLRs are single-span transmembrane receptors, and they have their name from the homology with the Toll gene in *Drosophila melanogaster [28]*. Up until now there are 14 mammalian TLRs identified, with 10 of them found in humans. Each TLR recognize different subsets of highly conserved PAMPs derived from viruses, bacteria, protozoa and fungi. Their localization in the cell coincides with the type of ligand they recognize [8].

1.3.1 Toll-like receptors on rheumatoid arthritis effector cells

The key-effector cells of RA, the SFs, are identified as innate immune cells. The literature reporting SFs' TLR expression is not cosistent. There is agreement that SFs express TLR1-6 [5], but certain articles also state the presence of TLR7 [8, 29]. When the TLRs bind their ligands, the expression of inflammatory mediators like CAMs, cytokines, chemokines and MMPs increases [5, 30]. TLR1, TLR2, TLR4, TLR5 and TLR6 are found in the plasmamembrane, and thus bind to surface associated PAMPs. In contrast, TLR3 and TLR7 are located in endosomal membranes and respond to internalized PAMPs [31]. More specifically, TLR1, TLR2 and TLR6 bind bacterial lipoproteins, such as peptiodoglycan (PGN). Dimerization of TLR2 with either TLR1 or TLR6 gives further ligand specificity. Moreover, TLR4 senses bacterial lipopolysaccharide (LPS), while TLR5 has the flagellar protein, flagellin, as its ligand. The endosomal TLR3 and TLR7 both respond to ribonucleic acid (RNA) from viruses. TLR3 recognizes double stranded RNA, whereas TLR7 binds single stranded RNA [8, 31]. Table 1.3.1 gives an overview of the TLRs expressed by SFs and the PAMPs they recognize. The table includes DAMPs and synthetic agonists, which also can activate TLRs.

Table 1.3.1 An overview of the PAMPs and DAMPs the different TLRs recognize, including examples of synthetic agonists [32].

Receptor	PAMPs	DAMPs	Synthetic agonists
TLR2 (dimerization with TLR1 or TLR6)	Bacterial lipoproteins	Heat shock proteins	Pam3CSK4 (TLR1/TLR2) FSL-1 (TLR2/TLR6)
TLR3	Viral dsRNA	dsRNA from necrotic cells	Poly(I:C)
TLR4	Bacterial LPS	Heat shock proteins, fibrinogen or hyaluronan	LPS (natural)
TLR5	Flagellin	-	Flagellin (natural)
TLR7	Viral ssRNA	-	Imidazoquinolines

The SFs are reported to have especially high levels of functional TLR2, TLR3, and TLR4 [5]. There are data suggesting that ligand binding by these TLRs leads to increased RANKL expression by RA SFs, and thus enabling osteoclast differentiation [15, 33]. Moreover, studies have shown that RA SFs highly express TLR3 and TLR4 [34], and these cells have especially high levels of TLR2 where cartilage and bone destruction occurs [35]. When compared to non-inflammatory cells, TLR2, TLR3 and TLR7 are found to be significantly elevated in RA SFs [8]. Furthermore, macrophages in the synovial tissue show increased expression of TLR2 and TLR4 in RA [9].

Early analysis of RA synovial tissue showed presence of PGN, bacterial deoxyribonucleic acid (DNA), and viral DNA [36, 37]. It was therefore hypothesized that infection may be the underlying reason for RA development. Due to the fact that healthy joints were found to have comparable levels of pathogenic molecules [38], this remains a highly debated hypothesis.

1.3.2 Toll-like receptors and endogenous molecules

As previously mentioned, it has been discovered that TLRs can recognize DAMPs. Endogenous molecules that are released during cell necrosis or components of the extracellular matrix that are up-regulated after tissue damage, are categorized as DAMPs. Upon tissue injury, DAMPs are essential factors necessary to initiate inflammatory responses in the absence of infection. Nonetheless, DAMPs have been linked to the pathophysiology of inter alia autoimmune diseases [8]. Heat shock proteins (HSPs), fibrinogen and hyaluronan are examples of DAMPs that are commonly found in inflamed joints. These can bind to TLR2 and TLR4, consequently activating cells. The synovial fluid of RA patients has also been shown to contain double stranded RNA from necrotic cells, and can activate e.g. RA SFs by binding to TLR3 [5]. Studies regarding this topic have shown that activation of TLR3 in RA SFs by endogenous RNA from necrotic cells, results in IL-6, IFN-β, and Th1-associated chemokine expression [30]. Moreover, evidence points towards DAMPs having different ways of activating TLRs and different resulting immune responses than PAMPs [8]. Endogenous TLR ligands are, as indicated, released due to the inflammatory response in RA. The ligands may be some of the contributing factors in perpetuating the disease, as demonstrated in figure 1.3.1 [9].

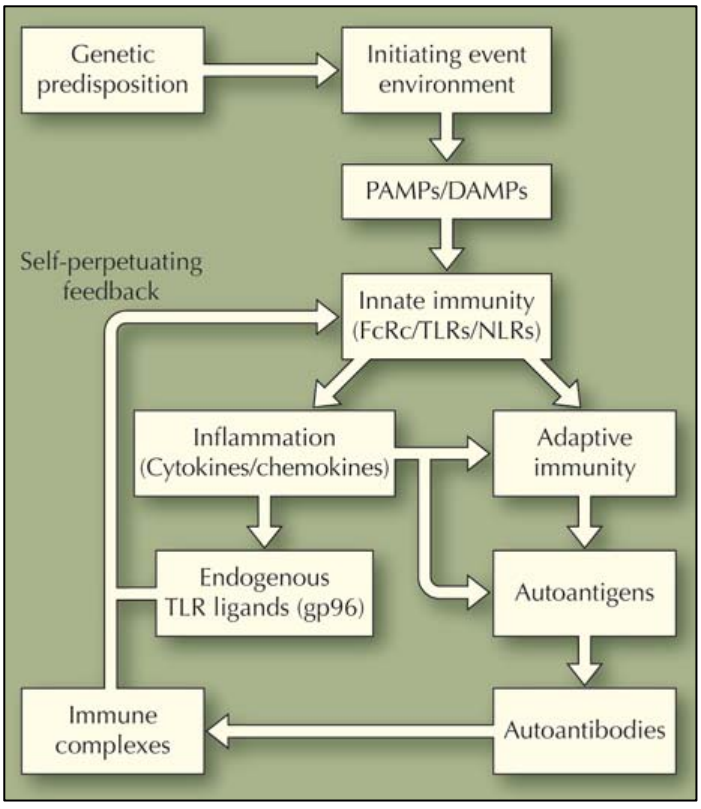

Figure 1.3.1 A proposed connection between the processes and molecules involved in RA pathogenesis. It is likely that endogenous TLR ligands and immune complexes are the main factors driving the self-perpetuating inflammatory response [9]. DAMP, damage associated molecular pattern; FcR, receptor for the Fc portion of IgG; gp96, endoplasmic reticulum stress response protein; NLR, Nod-like receptor; PAMP, pathogen associated molecular pattern; TLR, Toll-like receptor

1.3.3 The signaling of Toll-like receptors

All the mammalian TLRs have a common structure, with amino-terminal leucine-rich repeats as ligand binding domain and a carboxy-terminal Toll/IL-1 receptor (TIR) homology domain conveying the signal. When a ligand binds, conformational changes in the TLRs lead to activation of a signaling cascade resulting in transcription of genes involved in inflammatory responses initiated to destroy the recognized invaders [8, 31]. As shown in figure 1.3.2, different TLRs activate different signaling cascades. Most of them utilize the myeloid differentiation primary-response protein-88 (MyD88)-dependent pathway that activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs). This activation ultimately leads to the production of cytokines and other inflammatory mediators. In contrast to the other TLRs, TLR3 uses a MyD88-independent pathway, which TLR4 also is able to employ. This pathway results in expression of another set of genes via the transcription factor interferon regulatory factor-3 (IRF3). The genes activated by IRF3, such as IFN-α and IFN-β, are involved in inducing an anti-viral state in the cell [9, 31].

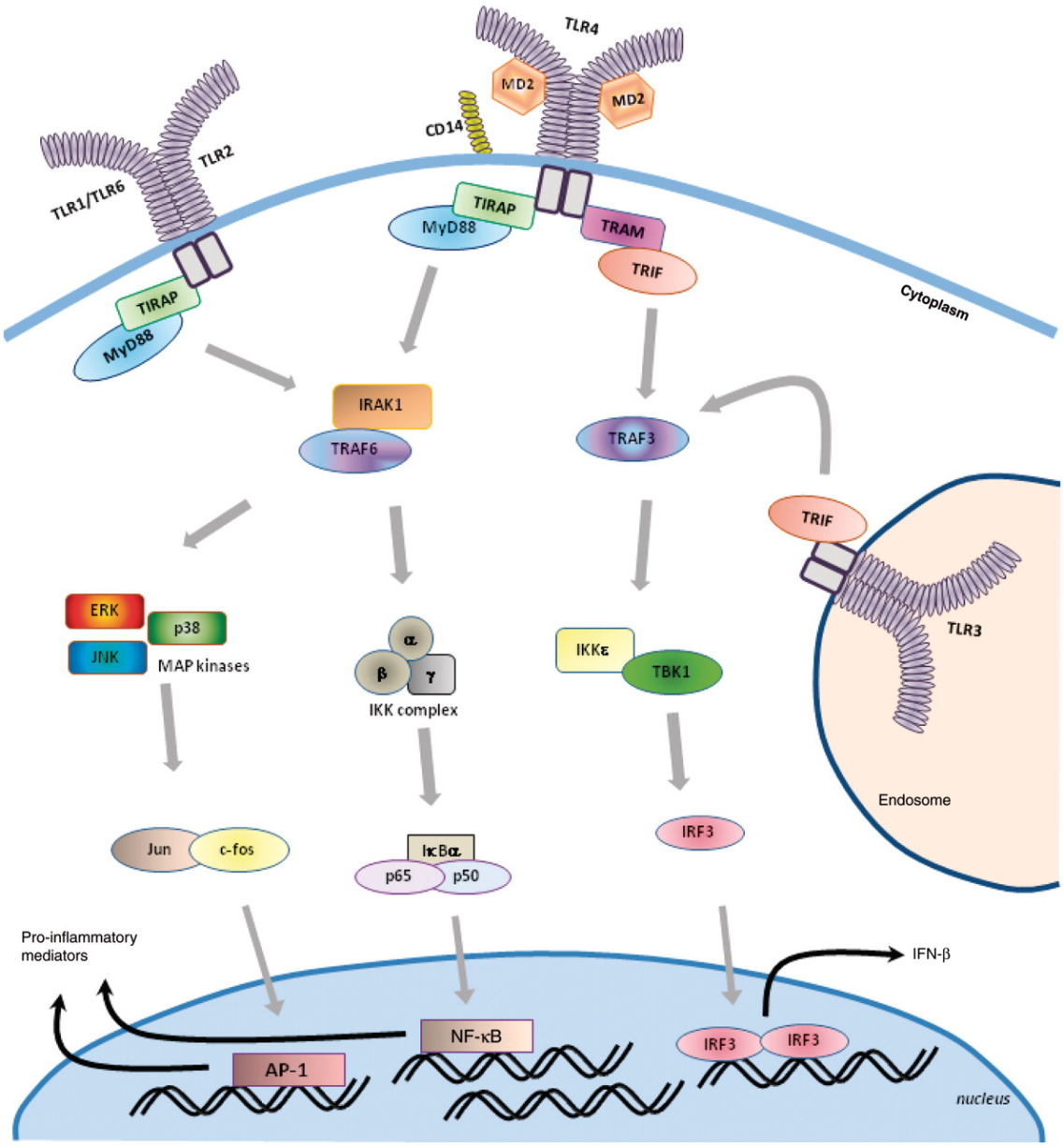

Figure 1.3.2 A simplified illustration of the signaling cascades set off by the different TLRs upon ligand binding [8]. AP, activating protein; CD, cluster of differentiation; ERK, extracellular-signal regulated kinase; IFN, interferon; IKK, I-kappa B kinase; IκBα, inhibitor of NF-κB; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon regulatory factor; JNK, c-jun N-terminal kinase; MAP, mitogen-activated protein; MD, myeloid differentiation protein; MyD88, myeloid differentiation primary-response protein-88; NF-κB, nuclear factor-κB; TBK, TANK-binding kinase; TIRAP, TIR domain-containing adaptor protein; TLR, Toll-like receptor; TRAF, TNF-α receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor-inducing IFN- β

1.4 Phospholipase A_2 in bone metabolism and inflammation

Eicosanoids are a group of molecules considered to be important lipid mediators [39]. These molecules have both physiologic and pathologic roles in the skeletal metabolism [40]. Eicosanoids are important in inflammatory responses, and are also linked to autoimmune diseases, such as RA [41]. These molecules are subdivided into three major groups: 1) prostanoids, which include prostaglandins (PGs) and thromboxanes (TXs), 2) leukotrienes, lipoxins, and hydroxy-fatty acids, and 3) epoxy and omega derivatives. The grouping corresponds to the pathways that the molecules are synthesized via. Eicosanoids can be derived from the 20-carbon omega-6 polyunsaturated fatty acid called arachidonic acid (AA). AA is normally esterified in membrane glycerophospholipids, but is released by the action of certain enzymes when needed. These enzymes are named phospholipase A_2 (PLA_2), and they are specific for hydrolysis of fatty acid ester bonds in the *sn*-2 position of membrane phospholipids [40].

The release of the 18-carbon omega-9 monounsaturated oleic acid (OA), together with AA, has been found in elevated levels upon stimulation in certain cell models. In these systems the OA release was due to $sPLA_2$ and $iPLA_2$ [42, 43]. OA has been shown to activate diverse pathways of immune competent cells and is often described as an anti-inflammatory mediator [44].

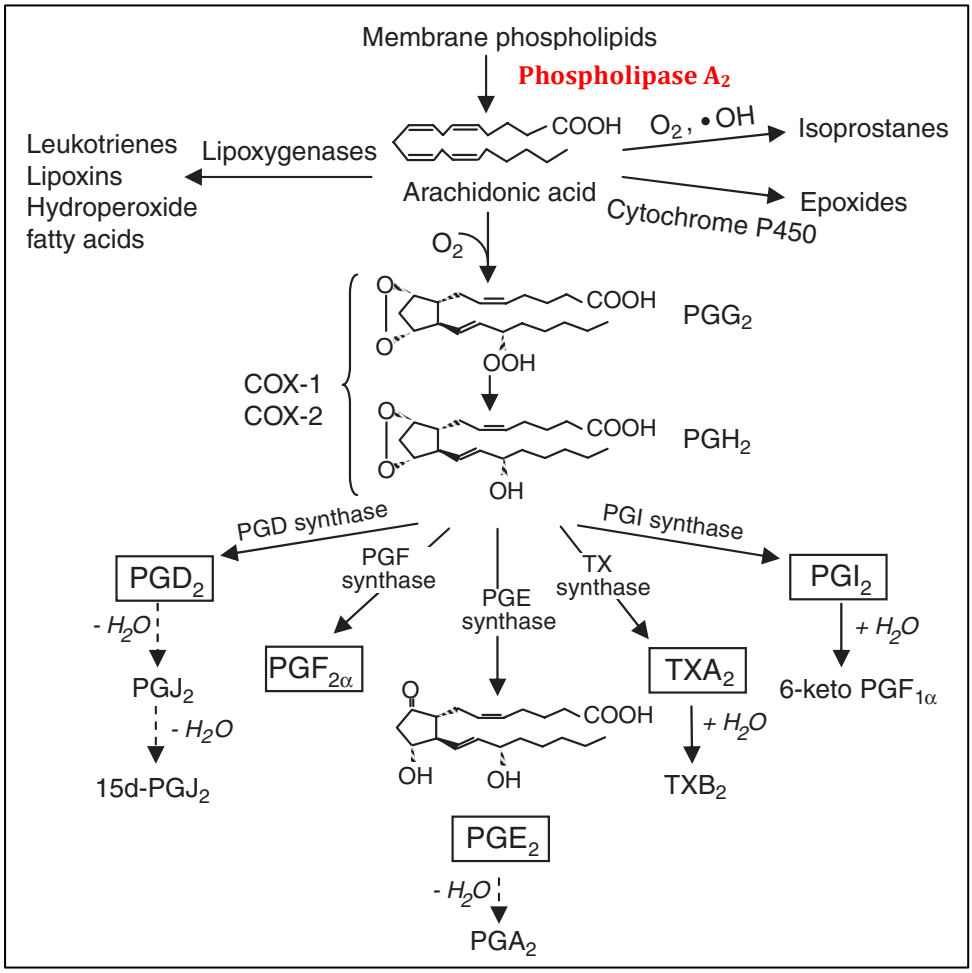

Figure 1.4.1 Pathway for synthesis of prostanoids, involving COX-1, COX-2, and different specific synthases [40]. The pathways of the other groups of eicosanoids are not shown in detail. COX, cyclooxygenase; PG, prostaglandin; TX, thromboxanes

1.4.1 Prostaglandin E_2 is a potent activator of bone resorption

The prostanoids are of the most studied eicosanoids in relation to bone metabolism. They are synthesized from AA via the cyclooxygenase (COX) pathway carried out by the enzymes COX-1, COX-2 and different synthases, as shown in figure 1.4.1 [40].

Bone tissue normally express high levels of PGs, but they are often found to be even more elevated in inflammatory conditions, such as RA [40]. Early studies showed that the E series of PGs are the most potent activators of bone resorption in organ culture [45], and PGE_2 is extensively studied within the field of osteology. In the body, exogenous PGE_2 seems to induce both bone formation and resorption. However, the extent of each process is varying [40]. This means that bone loss is observed in some models [46], while formation of bone is seen in others [47]. These complex effects on bone are probably due to the many PGE_2 receptors, EP1-EP4. Consequently, PGE_2 has the ability to activate various signaling pathways. Different receptors are found in both osteoblasts and osteoclasts [48, 49]. This complexity has made it difficult to determine the exact effects of endogenously PGs on bone remodeling, both under normal conditions and in disease. Nonetheless, many of the pro-inflammatory cytokines stimulate the expression of COX-2, and consequently the production of PGE_2. Since PGE_2 is a potent activator of bone resorption, it is blamed for contributing to the bone erosion seen in inflammatory diseases [40]. In addition, PGE_2 is shown to induce differentiation of osteoclasts by down-regulating the OPG expression of osteoblasts [50].

1.4.2 The secreted and the cytosolic phospholipase A_2

PLA_2s, are gathered in a superfamily consisting of 15 groups. There are also a number of subgroups corresponding to the different structures and mechanisms of the enzymes [51]. There are four distinct types of PLA_2 identified, namely the cytosolic PLA_2 ($cPLA_2$), the secreted PLA_2 ($sPLA_2$), the Ca^{2+}-independent PLA_2 ($iPLA_2$), and the lipoprotein-associated PLA_2 ($LpPLA_2$). Each different type of PLA_2 functions in lipid signaling, and has been linked to inflammatory diseases. There is therefore an increased interest in research concerning development of different PLA_2 inhibitors [52].

The $sPLA_2$ and the $cPLA_2$ are considered essential for the production of prostanoids [40]. There are several $sPLA_2$ groups, and group IIA $sPLA_2$ (GIIA $sPLA_2$) have been found in high concentration in synovial fluid of RA patients [52]. Prof. Berit Johansen, currently the head of the PLA_2 research group, participated in isolation, cloning and sequencing of the first discovered human, non-pancreatic PLA_2 present in platelets and in rheumatoid synovial fluid [53]. It had characteristics similar to GII PLA_2 isolated from snake venom, and was subsequently classified as GIIA $sPLA_2$. It has later been demonstrated that different $sPLA_2$s are present in RA and increase the production of PGs in cultured synovial cells. For the $sPLA_2$s to carry out the catalysis, they need millimolar concentrations of Ca^{2+}, and they have no AA selectivity [52].

There are different groups of the $cPLA_2$s, but group IVA $cPLA_2$ (GIVA $cPLA_2$) is the most studied. In contrast to the $sPLA_2$, the GIVA $cPLA_2$ is selective for AA release at the *sn*-2 position, and it is considered to be the most important $cPLA_2$ for freeing AA. Its expression can be increased by PGs and inhibited by IL-4, but most cells express this enzyme constitutively. The GIVA $cPLA_2$ also require Ca^{2+}, not for catalysis, but for translocation to membrane surfaces [40]. The enzyme is subsequently activated by phosphorylation at serine residues [41]. The GIVA $cPLA_2$ can be activated by

MAPKs like extracellular-signal regulated kinase (ERK) 1/2 and p38. Certain TLRs, for instance TLR4 on RA SFs, activate these MAPKs upon ligand binding [41]. It is established that GIVA $cPLA_2$ is central in inflammatory diseases because of its extensive release of AA, which in turn are converted into components that induce pain and inflammation [52]. In fact, studies on GIVA $cPLA_2$-deficient mice are showing that prevention of AA metabolism leads to weakening of the RA symptoms [54].

Prof. Berit Johansen and co-workers have shown that $cPLA_2$ activates the transcription factor NF-κB in response to TNF-α and IL-1β in human keratinocytes. Phosphorylation of $cPLA_2$ was found to be dependent on $sPLA_2$ and 5-lipoxygenase activities, which subsequently regulate the release of AA [55]. Furthermore, it was demonstrated that atypical λ/ι protein kinase C (λ/ιPKC) is involved in this signaling cascade. The λ/ιPKC was found to function downstream of $sPLA_2$ and 5-lipoxygenase, and it promotes $cPLA_2$ phosphorylation in a phosphatidylinositol 3 (PI3) kinase-dependent manner [56].

1.4.3 Phospholipase A_2 inhibitors

Which PLA_2 class that are targeted for inhibition, depends on the type of inflammatory disease. GIVA $cPLA_2$ is one of the targets in RA. However, it is likely that several groups are involved in chronic diseases, possibly at different stages of the disease progression. By using selective PLA_2 inhibitors, the role of each PLA_2 can be further explored. The hope is that PLA_2 inhibitors can treat inflammatory diseases such as RA. However, no selective PLA_2 inhibitors are currently available for treating patients [52]. Nonetheless, animal experiments have shown promising results. For example, Tai and co-workers have developed a GIVA $cPLA_2$ inhibitor and tested it on mouse models of collagen-induced arthritis (CIA). They found that arthritis was prevented if the inhibitor was given before the onset of disease. In mice already affected by arthritis, the inhibitor showed anti-arthritic activity by reducing the eicosanoid production [57]. Similar results of CIA models were recently obtained using novel GIVA $cPLA_2$ inhibitors characterized in Prof. Berit Johansen's lab (manuscripts in preparation). Tai and co-workers also detected a decreased expression of certain MMPs and COX-2 mRNAs as a result of GIVA $cPLA_2$ inhibition [57]. The PLA_2 research group has seen similar effects in the synovial SW982 cells [58].

1.5 The SW982 cell line as a model for synovitis in RA

The SW982 cells have their origin from a synovial sarcoma. At a clinic in Texas in 1974, this specific sarcoma was surgically removed from a Caucasian woman in her mid-twenties. A. Leibovitz subsequently initiated the SW982 cell line from the surgical specimen. In 1982, a sample of this cell line was sent to the American Type Culture Collection (ATCC), where these cells can now be purchased [59]. The SW982 cells are assumed to be type B synoviocytes (SFs) due to the presence of vimentin and smooth muscle actin, and the absence of CD11B [60].

The advantage of using a cell line, in contrast to primary mammalian cells, is that they do not possess a limited replicative life span and do not exhibit signs of senescence. Moreover, obtaining samples from RA synovium is a complicated process [61]. Phenotypically the SW982 cells have been shown to express the pro-inflammatory cytokines IL-1β, IL-6, and TGF-β. Furthermore, the cells express intercellular adhesion molecule-1 (ICAM-1), COX-2 and certain MMPs. All these genes show a highly increased expression when the cells are treated with IL-1β [62]. The PLA_2 research group has verified the presence of all these phenotypic characteristics [58]. In addition, the group have demonstrated that the SW982 cells express several PLA_2 isoforms: $sPLA_2$, $cPLA_2$, and $iPLA_2$ (manuscript in preparation).

1.6 Gene expression analysis by real-time quantitative polymerase chain reaction

A conventional polymerase chain reaction (PCR) results in end-point detection, and is used when a yes/no answer regarding the presence of PCR products is sufficient. In contrast, quantitative PCR (qPCR) is a method that enables researchers to monitor the amplification in real-time, thus making it possible to quantify specific nucleic acid sequences. The qPCR can be used in e.g. determination of viral load, detection of genetically modified organisms, single-nucleotide polymorphism (SNP) genotyping, and allelic discrimination. Moreover, it is commonly applied in gene expression analysis, which is the area of application in this master's thesis. For this purpose RNA templates are used, but first the RNA is transcribed into complementary DNA (cDNA) by the enzyme reverse transcriptase [63].

1.6.1 Detecting qPCR products using SYBR Green I

The detection of products in qPCR is based on fluorescence measurements during each cycle of the reaction - the amount of fluorescence is proportional to the amount of PCR product. There are several detection chemistries available for use in qPCR, such as fluorescent dyes and fluorescently labeled sequence-specific probes. The one used in this thesis is the fluorescent dye SYBR Green I. This dye emits fluorescence only when bound to double-stranded DNA molecules, as shown in figure 1.6.1. This means that the detection will occur during the extension steps of the reaction and the signal will increase as the qPCR products accumulate. The advantage of using SYBR Green I is that many different targets can be detected, and it is not necessary to make target-specific probes. Nonetheless, it is important that the primers used in the reaction are highly specific due to the risk of obtaining fluorescence from nonspecific products [63].

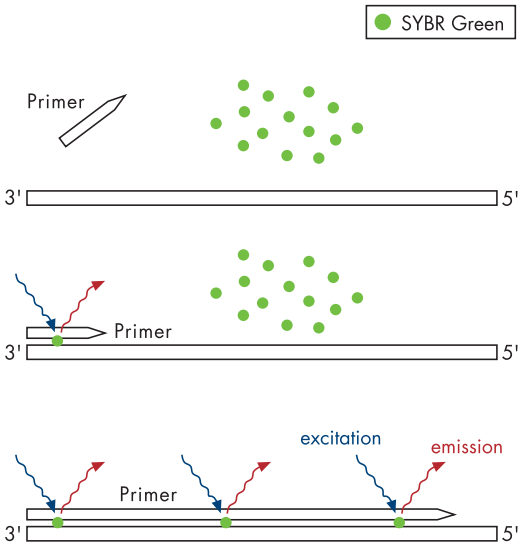

Figure 1.6.1 Detection of qPCR products by the fluorescent dye SYBR Green I [63].

1.6.2 The $\Delta\Delta C_q$ method for relative quantification

Nucleic acids can be either absolutely or relatively quantified after performing a qPCR. Relative quantification is commonly used for gene expression analysis. In this case the ratio between the amount of target and the amount of an endogenous reference molecule, commonly a housekeeping gene, is determined. It is important that the expression level of the reference is constant under all experimental conditions. If this is the case, the ratio of different samples can be used for comparison of gene expression [63].

The choice of approach for relative quantification is based on whether the target and the reference gene are amplified with comparable or different efficiencies. If the amplification efficiencies are comparable, you can safely use the comparative method called the $\Delta\Delta C_q$ method. Quantification cycle (C_q), also known as threshold cycle (C_T), is a value denoting at which cycle the amplification plot crosses the threshold. The threshold is set within the log-linear phase of the amplification curve - above the background fluorescence baseline, but significantly below the plateau of the curve, as shown in figure 1.6.2 [63].

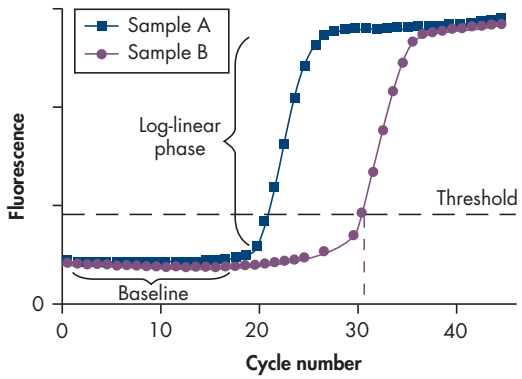

Figure 1.6.2 Examples of amplification curves with the terms used in qPCR [63].

Before applying the $\Delta\Delta C_q$ method, amplification efficiencies must be compared. To compare the amplification efficiencies, a standard curve is generated. This is made by plotting the difference between the C_q value of the target gene and the C_q value of the reference gene, ΔC_q, against the logarithm of input amount of RNA. The amplification efficiencies are considered comparable if the slope of the linear regression line is < 0.1. If this is the case, it is not necessary to generate standard curves in following experiments [63]. The change in gene expression of different samples (e.g. stimulated cells) relative to a calibrator sample (e.g. untreated cells) can then be calculated by the following steps [64]:

1. Calculate ΔC_q for each sample, including the calibrator sample, to normalize for the amount of template used:

$$\Delta C_q \text{ (sample)} = C_q \text{ target gene} - C_q \text{ reference gene} \quad (1)$$

$$\Delta C_q \text{ (calibrator)} = C_q \text{ target gene} - C_q \text{ reference gene} \quad (2)$$

2. Normalize all samples to the calibrator sample by determining the $\Delta\Delta C_q$:

$$\Delta\Delta C_q = \Delta C_q \text{ (sample)} - \Delta C_q \text{ (calibrator)} \quad (3)$$

3. Calculate the normalized gene expression level of the target gene relative to the calibrator, referred to as fold change:

$$\text{Fold change} = 2^{-\Delta\Delta Cq} \quad (4)$$

If the fold change of a target gene has a value of e.g. 4 in stimulated cells, it means that this gene is 4 times up-regulated in these cells relative to the untreated ones. In contrast, if the fold change of the target gene in the sample of interest has a value lower than in the calibrator sample (fold change = 1), it means that the gene is down-regulated accordingly.

1.6.3 Analysis software

For easier handling of big data sets, analysis of the qPCR results can be done using appropriate computer software. In this master's project LinRegPCR, developed by Ramakers and co-workers, was used. This is a program that performs regression analysis of imported raw fluorescence data. The program defines the baseline and performs a baseline correction, before it computes the amplification efficiencies. Then the C_q value is determined from the exponential phase of PCR amplification and the starting amount of cDNA can be calculated [65]. If relative quantification is desired, the C_q values from LinRegPCR can for example be entered into the relative expression software tool REST 2009 from Qiagen, as was done in this thesis. REST performs reference gene normalization and provides relative gene expression like the $\Delta\Delta C_q$ method does, but it also takes the amplification efficiency into account. In addition, the software performs statistical analysis. Two groups, treated vs. control, are compared and tested for significant difference by using a randomization test [66].

1.7 The aim of the thesis

An ideal way of treating autoimmune diseases like RA would be to weaken only the autoimmune response, while not interfering with the rest of the immune system. This has proven to be very difficult [7]. The existing drugs for RA are basically divided into two major groups; non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs). The purpose of the NSAIDs, such as ibuprofen, is to treat the symptoms by reducing PG synthesis through COX inhibition [3]. DMARDs, on the other hand, have various mechanisms of action. There are biological DMARDs aiming to e.g. inhibit TNF (adalimumab) or block IL-6 signaling (tocilizumab) [1]. In addition, there are non-biological DMARDs, such as methotrexate that exerts immunosuppressive effects through interference with DNA synthesis and cell replication [67]. In contrast to NSAIDs, the DMARDs can hinder disease progression by preventing pain, swelling and bone destruction [3]. Due to still unknown factors of RA pathogenesis and the heterogeneity of the disease, a treatment that gives remission in the majority of patients has not yet been found – and much less a cure. This means that a continued search for new treatment principles and drugs is necessary [1].

As stated earlier, PLA_2 enzymes have a central role in production of inflammatory mediators. Thus, controlling the activity of PLA_2 constitutes an attractive therapeutic target for treating conditions like RA. As of today, not much research has focused on the effect of PLA_2 inhibition on downstream cellular processes in bone metabolism. One of the main goals of this master's thesis will therefore be to explore the possibility of $cPLA_2$ being an upstream regulator of the pathways involving RANKL, OPG, and DKK1. This can give an indication on whether inhibiting $cPLA_2$ will be beneficial to reestablish the bone homeostasis. Inhibition of $cPLA_2$ could potentially address some of the underlying causes of RA, i.e. act as a DMARD. If effective, $cPLA_2$ inhibitors would function on an earlier stage than NSAIDs. The $cPLA_2$ enzyme is a target of investigation with the aim of finding a drug that is more efficient and have less side effects [68]. Furthermore, little is known regarding $cPLA_2$'s involvement in signaling via TLRs, at least not in association with RA. Another goal of this thesis will be attempting to establish the SW982 cells as a model system for TLR signaling by characterizing their expression of the TLRs known to be present in SFs. It is also of interest to explore the possibility of a connection between TLRs and the PG pathway. This will be groundwork for further research regarding the possible importance of $cPLA_2$ in TLR signaling in these cells.

Based on this introduction chapter and the statements above, the following questions have been deduced and will be pursued experimentally:

1) Do the SW982 cells express RANKL, OPG, DKK1 and TLR1-TLR7?

2) Do TLR agonists affect the expression levels of RANKL, OPG and DKK1 in SW982 cells? Are the $cPLA_2$ and COX enzymes involved?

3) Do TLR agonists affect the activity of PLA_2s?

4) Are RANKL, OPG, DKK1 and TLR1-TLR7 expression in SW982 cells affected by the pro-inflammatory cytokine TNF-α? Is $cPLA_2$ involved?

2. MATERIALS & METHODS

2.1 Reagents, solutions and materials

All reagents, solutions, and materials used during this master's project will be listed here.

Cell culture and cell experiments

SW982 cells were obtained from the ATCC. 75 cm^2 and 175 cm^2 cell culture flasks and cell scrapers were from Sarstedt. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Gentamicin solution, dimethyl sulfoxide (DMSO), and fatty acid free bovine serum albumin (fBSA) were all purchased from Sigma-Aldrich. Both fetal bovine serum (FBS) and 0.05% trypsin-EDTA were obtained from Gibco, while phosphate buffered saline (PBS) was from Oxoid. TNF-α (10 µg/ml) was purchased from R&D Systems and IL-1β (2 µg/ml) from Roche. The 6-well and 48-well plates were from Corning. The TLR agonists FSL-1 and Pam3CSK4 were obtained from InvivoGen, while Poly(I:C) and LPS were received from another NTNU lab. Inhibitors used in the cell experiments, and the suppliers, are listed in table 2.1.

Table 2.1 An overview of the inhibitors used in this master's project, including suppliers and molecular targets.

Inhibitor	Supplier	Target
InhibX (real name not published)	Prof. George Kokotos, University of Athens	**cPLA$_2$**
AVX002	Synthetica Oslo	**GIVA cPLA$_2$** [69]
CAY10502	Cayman chemical	**GIVA cPLA$_2$** [70]
CAY10590	Cayman chemical	**GV sPLA$_2$** [71]
Varespladib (LY315920)	Selleck chemicals	**GIIA sPLA$_2$** [72]
BEL	Cayman chemical	**GVIA and GVIB iPLA$_2$** [73]
MAFP	Cayman chemical	**GVIA iPLA$_2$ + GIVA, GIVB and GIVC cPLA$_2$** [73]
Indomethacin	Sigma-Aldrich	**COX-1 and COX-2** [74]

RNA isolation

RNeasy Mini Kit and RNase-free DNase Set were bought from Qiagen, and β-mercaptoethanol from Sigma-Aldrich.

cDNA synthesis

5x first strand buffer, 0.1 M DTT, and moloney murine leukemia virus reverse transcriptase (M-MLV RT) were all obtained from Invitrogen. Deoxynucleotide triphosphate (dNTP) mix (10 mM) was purchased from Sigma-Aldrich, while both random primers and recombinant RNasin ribonuclease inhibitor were from Promega.

qPCR

SYBR Green JumpStart Taq ReadyMix was bought from Sigma-Aldrich. This mix contains 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP), stabilizers, 0.05 unit/µl Taq DNA Polymerase, JumpStart Taq antibody and SYBR Green I dye. RT^2 qPCR primer assays for human OPG, RANKL, DKK1, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, and 18SrRNA were all obtained from Qiagen. Primer pairs designed by the PLA_2 research group were purchased from Sigma-Aldrich: IL-6 fwd, IL-6 rev, COX-2 fwd, COX-2 rev, 18S rRNA fwd, and 18S rRNA rev. Non-skirted, natural colored 96-well qPCR plates and optically clear adhesive seal sheets were obtained from Thermo Scientific.

Gel electrophoresis

Agarose, Trizma base, ethylenediaminetetraacetic acid (EDTA), and acetic acid solution were all purchased from Sigma-Aldrich. GelRed nucleic acid gel stain was bought from Biotium, 6x Orange loading dye from Fermentas, and 100bp DNA ladder from Promega.

Enzyme-linked immunosorbent assay

DuoSet enzyme-linked immunosorbent assay (ELISA) for human OPG, reagent diluent, substrate solution and normal goat serum were all obtained from R&D Systems. The PBS, wash buffer (0.05 % Tween20 in PBS) and stop solution (0.5 M H_2SO_4) was self-prepared. The PBS was from Gibco, Tween20 from Cayman Chemical Company, and the H_2SO_4 from Sigma-Aldrich. Clear polystyrene flat-bottomed 96-well Nunc MaxiSorp microplates were purchased from eBioscience, while the aluminium seal films were from Corning.

Arachidonic and oleic acid release assay

AA[5,6,8,9,11,12,14,15-^{3}H(N)] 0.1 mCi/ml, OA[1-^{14}C] 0.1 mCi/ml and the scintillation cocktail OptiPhase Supermix were all purchased from Perkin Elmer. NaOH was from Sigma-Aldrich.

2.2 Cell culture and cell experiments

SW982 cells were cultivated using 75 cm^2 or 175 cm^2 cell culture flasks. The medium used was DMEM with 10% FBS, 0.3 mg/ml L-glutamine, and 0.1 mg/ml gentamicin added – hereafter referred to as 10% DMEM.

The cells were split in a sterile cabinet when they had reached a sub-confluent state, i.e. every 3-4 days. The split ratio varied from 1:3 to 1:6, depending on the density of the cells. The volumes presented here refer to the use of a 75 cm^2 cell culture flask. When cells were split, they were washed 2 times with 10 ml room temperate PBS. They were then incubated with 1.5 ml of preheated (37 °C) 0.05% trypsin-EDTA for 2 minutes at 37 °C. The flask was tapped against the lab bench for better loosening of the cells. To deactivate the trypsin, 4x volume of 10% DMEM was added. The whole solution was then transferred to a 15 ml tube and centrifuged at 700 rpm at 25 °C in 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 3-6 ml 10% DMEM, depending on the split ratio. 1 ml of the cell suspension was put back in the culture flask with 9 ml fresh, preheated (37 °C) 10% DMEM. The culture was kept in a humidified CO_2 incubator at 37 °C.

When experiments were to be performed, the remainders of the resuspended cells were counted using a Bürker chamber and the cell suspension was diluted to obtain the appropriate cell amount for the type of experiment to be executed. Every step where 10% DMEM is used, the medium is preheated to 37 °C. The same goes for DMEM with only L-glutamine and gentamicin added (no FBS) – referred to as serum-free DMEM (SF-DMEM).

2.2.1 Cell experiments for qPCR

Stimulation with TLR agonists

2 ml 10% DMEM was added to the wells in 6-well plates, and 1 ml with 300 000 cells was seeded in each well. The cell plates were placed in the incubator to grow confluent. They were incubated 3 additional days after reaching 100% confluence (~ 30 hours after seeding), i.e. 3 days post-confluence. At 2 days post-confluence the cells were starved, meaning that the 10% DMEM in each well was replaced by 1.5 ml SF-DMEM. The following day the cells were stimulated. Old SF-DMEM was removed, and 1.5 ml fresh SF-DMEM was added to the control wells. In the test wells, 1.5 ml of the respective TLR agonist solution was added. TLR agonist concentrations used for stimulation were: 100 ng/ml of FSL-1, Pam3CSK4 (P3C), and LPS, and 5 µg/ml Poly(I:C) (PIC). 1.5 ml of 10 ng/ml IL-1β solution was added to a well as a positive control. If inhibitors were used, the cells were pre-incubated with 1.5 ml inhibitor solution for 2 hours. Inhibitors used were InhibX, AVX002 and Indomethacin - all with a concentration of 5 µM. After the pre-incubation, 375 µl of the TLR agonists with 5x the concentrations above was added. The plates were left in the incubator for the desired time period (varied from 3-24 hours).

Stimulation with TNF

1 ml with 300 000 cells was seeded evenly in each well of four 6-well plates with 2 ml 10% DMEM per well. Again, the cells were incubated until reaching 3 days post-confluence, including starvation with 1.5 ml SF-DMEM per well at 2 days post-confluence. Old SF-DMEM was removed and replaced by 1.5 ml fresh SF-DMEM. The $cPLA_2$ inhibitors, InhibX and AVX002, were diluted in SF-DMEM to a 5 µM and 1 µM concentration and 1.5 ml of each was added to separate test wells. After adding the inhibitors, the cells were pre-incubated for 2 hours. Then 375 µl of 50 ng/ml TNF-α was added to the TNF-α well and to each of the inhibitor wells. Together these wells now contained 1875 µl, i.e. the final TNF-α concentration was 10 ng/ml. Lastly, 375 µl of additional SF-DMEM was added to the control wells. The four plates were then left in the incubator for 6, 12, 24 and 48 hours respectively.

2.2.2 Cell experiments for arachidonic and oleic acid release assay

0.5 ml with 50 000 cells was seeded in each well of 48-well culture plates. Note that cells were not seeded in the wells of the uttermost row. I.e., only the inner 24 wells were used, due to risk of edge effect. 0.5 ml PBS was added to the uttermost wells. At 3 days post-confluence, the cells were radioactively labeled with 0.1 mCi/ml AA[^{3}H] and 0.1 mCi/ml OA[^{14}C]. Labeling medium was made by mixing SF-DMEM with 3 µl/ml AA[^{3}H] and 0.6 µl/ml OA[^{14}C]. Old 10% DMEM in the 48-well plates was removed. 160 µl of the labeling medium was then added to each well, and the plates were incubated for 16-20 hours. The labeling medium was aspirated and the unincorporated labeled fatty acids were removed by washing with 300 µl preheated PBS/0.2% fBSA (2 ml of 5% fBSA stock per 50 ml PBS). The cells were subsequently washed with 300 µl preheated PBS. When stimulating with TLR agonists alone, the PBS was replaced by 200 µl of the respective TLR agonist solution and 200 µl SF-DMEM in the control wells. Different TLR agonist concentrations were tested: 100 ng/ml vs. 50 ng/ml of FSL-1, 300 ng/ml vs. 100 ng/ml of P3C, and 5 µg/ml vs. 1 µg/ml of PIC. 200 µl of 10 ng/ml IL-1 solution was added to wells as positive control. All treatments were performed in triplicates. The cell plates were incubated for the desired time period (varied from 3-24 hours).

When using inhibitors, however, the triplicates were treated with 160 µl of the respective inhibitor solution and pre-incubated for 2 hours. Inhibitors used were AVX002, CAY10502, CAY10590, Varespladib, BEL and MAFP. AVX002 were used with a concentration of 5 µM, CAY10502 was 1 µM and the others were 10 µM. After the pre-incubation, 40 µl of the TLR agonists with 5x the desired concentrations was added. Final concentrations were: 50 ng/ml of FSL-1, 300 ng/ml of P3C, and 1 µg/ml of PIC. The cells were then left in the incubator for 9 hours.

2.3 Collection of culture supernatants, lysis and RNA isolation

There are different ways of isolating RNA. In this master's project the RNeasy Mini Kit from Qiagen was used. This kit utilizes selective binding of silica-based membrane and microspin technology. The buffers in this kit provide a high-salt system, which enables the silica membrane to bind up to 100 µg of RNA longer than 200 bases. By the use of a buffer containing the denaturing agent guanidine-thiocyanate, the cells are first lysed and homogenized. To ensure that the RNA molecules are kept intact, RNases are inactivated by guanidine-thiocyanate. The samples are then mixed with ethanol and added to spin-columns containing the silica-based membrane, where the ethanol will provide the right binding conditions. Contaminants, such as proteins, cellular debris, genomic DNA etc., are washed away in subsequent washing steps by adding different wash buffers and centrifuging. Lastly, the purified RNA molecules are eluted [75].

After the cells in 6-well plates from section 2.2.1 had been incubated for the desired time period, the cell culture supernatant was removed. The supernatants of interest were collected and placed in -80 °C for storage until further analysis. The cells remaining in the wells were subsequently lysed. For the lysis, Buffer RLT (lysis buffer) and β-mercaptoethanol (10 µl per ml Buffer RLT) were mixed. 350 µl of this mixture was then added in each well and left to take effect for ~2 minutes, before the cells were scraped off using cell scrapers. Lysed cells were transferred to eppendorf tubes, vortexed and stored at -80 °C until isolation of total RNA was performed.

For the RNA isolation procedure, the RNeasy Mini Kit spin protocol for animal cells was followed. Firstly, the cell lysates were thawed and 350 µl 70% ethanol was added to each sample to homogenize. Lysate and ethanol were thoroughly mixed by pipetting, before transferring 700 µl of each sample to an RNeasy spin column in a 2 ml collection tube. Lids were closed and the samples were centrifuged for 15 seconds. Flow-through was discarded, before adding 700 µl of Buffer RW1 to each RNeasy spin column. The samples were once more centrifuged for 15 seconds to wash the membrane in the column. Flow-through was discarded and 500 µl of Buffer RPE was added to each RNeasy spin column. Yet again, the samples were centrifuged for 15 seconds for washing. Flow-through was discarded and 500 µl of Buffer RPE was once more added to each RNeasy spin column. The samples were now centrifuged for 2 minutes for further washing. RNeasy spin columns were then placed in new 2 ml collection tubes and centrifuged at full speed for 1 minute. After this, each column was placed in 1.5 ml eppendorf tubes, and 30 µl of RNase-free water was directly added to each spin column membrane. The samples were incubated in room temperature for 5 minutes (not in the Qiagen protocol, but this has proved to increase the RNA yield) before they were centrifuged for 1 minute to elute the RNA. All the centrifugation steps were performed at 20–25°C with a speed of ≥ 10 000 rpm in a standard microcentrifuge. After the isolation procedure, the RNA concentration (ng/µl) and its purity were determined by adding 1.5 µl of the samples to a NanoDrop ND-1000 spectrophotometer. The RNA was subsequently stored at -80 °C.

The Qiagen RNeasy Mini handbook states that it is normally not necessary to perform DNase digestion when using RNeasy kit. The kit's silica-membrane technology is supposed to remove most of the DNA [75]. However, after some problems with one of the qPCR primers, an isolation protocol including on-column DNase digestion was used. The digestion was done with the RNase-free DNase Set and the steps differed

slightly from the above isolation procedure. The ethanol homogenizing and spin down were the same. Samples were then washed with 350 µl Buffer RW1 (15 seconds at ≥ 10 000 rpm), and flow-through was discarded. 80 µl DNase I incubation mix (10 µl DNase I stock solution mixed in 70 µl Buffer RDD) was added directly onto the spin column membrane, and incubated in room temperature for 15 minutes. Then the DNase was removed by washing with 350 µl Buffer RW1 (15 seconds at ≥ 10 000 rpm). From this point on, where the RPE Buffer washing step is started, the protocol is the same as the one above.

2.4 Synthesis of complementary DNA

Before the RNA samples, containing primarily mRNA, are used in qPCR, the RNA is converted into cDNA. It is not possible for eukaryotic cells to go from RNA and back to DNA. However, some viruses have an enzyme that can do this, and so the enzyme is called reverse transcriptase. Such an enzyme is utilized in the synthesis of cDNA. Furthermore, in this master's project random primers are used for the cDNA synthesis. This results in cDNA from all mRNA molecules, although not in full lengths [76].

The RNA samples were thawed. RNA concentration and purity were measured by NanoDrop once more to ensure that the measurements were similar to the ones prior to freezing. The concentration values were then used for calculating the amounts of RNA and distilled water (dH_2O) that were to be added in each cDNA synthesis reaction. For every reaction there should be 1 µg/µl RNA in a total of 20 µl.

All reagents were thawed (stored at -20 °C), vortexed and spun down before use. A mastermix was then prepared. The following amounts per reaction were used: 4 µl 5x first strand buffer, 2 µl 0.1 M DTT, 2 µl dNTP mix and 1 µl random primers (diluted to 100 µg/ml). Correct amounts of dH_2O and RNA were mixed in new 0.5 ml tubes. 1 µl of each of the enzymes recombinant RNasin and M-MLV RT was then added to the mastermix. The mastermix was vortexed, spun down and 11 µl was subsequently added to each tube with RNA and dH_2O. Samples were incubated in room temperature for 10 minutes, before incubation at 37 °C (heating block) for 1 hour. The reaction was terminated by incubating the samples at 95 °C (heating block) for 5 minutes. The cDNA samples were stored at -20 °C until further analysis.

2.5 Real-time quantitative polymerase chain reaction

As explained in the introduction chapter, qPCR can be used for various purposes where the aim is to quantify the amount of nucleic acids present in a sample. The qPCR is often used to analyze gene expression, which is the area of application in this master's project. To analyze the expression of certain genes, mRNA templates are used. However, before running the qPCR the mRNA has been transcribed into cDNA, as described in the last section.

Before starting the qPCR set-up, the cDNA samples were thawed, diluted 1:12 and kept on ice. Mastermixes for the target gene (OPG, RANKL, DKK1 or TLR1-TLR7) and the reference gene (18SrRNA) were prepared. Firstly, 6.5 µl dH_2O per reaction was pipetted into new tubes. 1 µl of the desired RT^2 qPCR primer assay (a mix of 2 primers, 10 µM each) per reaction was mixed into the dH_2O. The primer assays were thawed (stored at -20 °C), vortexed and spun down before use. Lastly, 12.5 µl of the SYBR Green JumpStart Taq ReadyMix per reaction was added to the mastermixes. Mastermixes were vortexed and spun down, before pipetting 20 µl in each well of a 96-well qPCR plate. 5 µl of the appropriate cDNA sample was subsequently added to the mastermix in each well. The plate was sealed and centrifuged for 1 minute at 1200 rpm. The qPCR was conducted on a Mx3000P cycler (Stratagene). Cycling conditions used were obtained from the handbook attached to the RT^2 qPCR primer assays [77]. The conditions started with an initial denaturation step at 95 °C for 3 minutes (shorter time than given in the handbook – optimized by the PLA_2 group) to activate the Taq DNA Polymerase. Then followed 40 cycles of 15 seconds at 95 °C (denaturation), 40 seconds at 55 °C (annealing) and 30 seconds at 72 °C (extension and fluorescence detection). These conditions were used for all the RT^2 qPCR primer assays, except for the DKK1 primer, where the annealing temperature was set to 60 °C instead of 55 °C. Mx3000P melting curve program (1 cycle of 95 °C for 1 minute, 55 °C for 30 seconds, and 95 °C for 30 seconds) was ran for dissociation curve analysis. The RT^2 qPCR primer assay details are listed in table 2.5.1.

Table 2.5.1 RT^2 qPCR primer assays used in this master's project. RefSeq accession # refers to the sequence used to design the RT^2 qPCR primer assay, and the reference position is the position of the amplicon in the RefSeq sequence. The details were obtained from the primer assay data sheets.

Gene	UniGene #	RefSeq accession #	Reference position	Product band size (bp)
OPG (TNFRSF11B)	Hs.81791	NM_002546.3	1373	157
RANKL (TNFSF11)	Hs.333791	NM_003701.3	2025	90
DKK1	Hs.40499	NM_012242.2	1016	183
TLR1	Hs.654532	NM_003263.3	586	189
TLR2	Hs.519033	NM_003264.3	437	154
TLR3	Hs.657724	NM_003265.2	2355	97
TLR4	Hs.174312	NM_138554.3	5431	68
TLR5	Hs.604542	NM_003268.5	2952	136
TLR6	Hs.662185	NM_006068.4	2784	151
TLR7	Hs.659215	NM_016562.3	318	195
18S rRNA	N/A	X03205.1	1447	100

In addition, primer pairs designed by members of the PLA_2 research group were used. The same approach as for the RT^2 qPCR primer assay was used when preparing mastermixes. However, there was a difference in reagent amounts: 0.75 µl of each of the forward and the reverse primer was added to 5.75 µl dH_2O, and lastly 12.75 µl SYBR Green JumpStart Taq ReadyMix was added. Cycling conditions were also different: An initial step at 95 °C for 3 minutes followed by 40 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 72 °C. The primer pairs and their details are listed in table 2.5.2.

Table 2.5.2 The primer pairs designed by the PLA_2 research group that were used in this master's project.

Gene	Sequence	Product band size (bp)
IL-6 fwd	5'-TGTGTGAAAGCAGCAAAGAG	105
IL-6 rev	5'-GCAAGTCTCCTCATTGAATCC	
COX-2 fwd	5'-GGGGATCAGGGATGAACTTT	153
COX-2 rev	5'-TGGCTACAAAAGCTGGGAAG	
18S rRNA fwd	5'-GTAACCCGTTGAACCCCATT	150
18S rRNA rev	5'-CCATCCAATCGGTAGTAGCG	

The resulting qPCR data were analyzed using LinRegPCR and REST 2009 software.

2.6 Gel electrophoresis

Gel electrophoresis is a method for separating DNA fragments, and agarose gels are the most commonly used. Agarose is a polymer consisting of agarobiose subunits. Upon gelation, the polymers form a network with a specific pore size, which is determined by the agarose concentration. The separation of DNA fragments is based on the fact that their phosphate backbone is negatively charged. Consequently, the fragments will migrate towards the positive anode when voltage is applied. The DNA molecules have a uniform mass/charge ratio, and they are therefore separated according to their size. The shortest fragments will travel furthest, because they will migrate faster through the pores of the agarose gel than the longer ones [78].

Before making the gel, tris-acetate-EDTA (TAE) buffer was prepared in a 50x stock solution. To prepare 1 liter, 242 g Trizma base, 57.1 ml glacial acetic acid, and 100 ml 0.5 M EDTA were dissolved in 1 liter deionized water. A 1x working solution was then prepared by mixing the 50x stock with water at a 1:4 ratio.

To make a 1.5% agarose gel, 100 ml 1x TAE was mixed with 1.5 g agarose powder, and the solution was heated until there were no visible agarose grains left. 12 µl of GelRed stain was added. When the solution was cold enough to handle, it was poured into a gel container. A comb with the desired number of spikes was placed in one of the ends, and the solution was left to solidify for about 30 minutes. While waiting, 13 µl of each qPCR product was mixed with 2.5 µl loading dye. 3 µl of the standard ladder was also mixed with loading dye. After the gel had solidified, the comb was removed and the gel was placed in an electrophoresis container. 1x TAE buffer was poured in, until the entire gel was covered. Standard ladder and qPCR products were added in the wells of the gel, which faced the side of the negative electrode. Electrophoresis was carried out at 100V for 45 minutes. The resulting bands on the gel were visualized using a Gel Doc machine (Bio-Rad).

2.7 Enzyme-linked immunosorbent assay

ELISA is a method used for detection and quantification of a specific protein, or another antigen of interest. The ELISA used in this master's project is called sandwich ELISA. This type of ELISA is based on the use of two antibodies that bind the target protein, but at different epitopes. The first antibody is used for coating the test wells. The sample is then added, before the second antibody is allowed to bind. This will result in a sandwich, where the target protein will be located between the two antibodies. The second antibody has an enzyme, directly or indirectly, attached. This enzyme produces a colored product from a colorless substrate, meaning that the color intensity is directly proportional to the amount of target protein present [79].

Sandwich ELISA was performed as advised in the R&D Systems kit protocol, except for some minor adjustments that will be stated here. To prepare the plates, the capture antibody was diluted to a working concentration of 2 µg/ml in PBS, and the 96-well microplate was immediately coated with 100 µl antibody solution per well. The plate was sealed and incubated overnight at room temperature. For longer storage, the plates were placed in 2-8 °C.

The samples used for analysis were frozen (-80 °C) cell culture supernatants collected after stimulation with TLR agonists, as described in section 2.2.1. Before use, the supernatants were thawed and centrifuged at 7000 rpm for 5 minutes at 4 °C. After the coating period, the wells in the coated plates were emptied and washed with 300 µl wash buffer. The washing was repeated 4 times, making it a total of 5 wash steps (instead of 3 steps with 400 µl as in the kit protocol). After the last wash, the plate was blotted against paper towels to remove remaining wash buffer. The plate was subsequently blocked by adding 300 µl reagent diluent to each well, sealing it and incubating for minimum 1 hour at room temperature. The 5 washing steps were then repeated before adding 100 µl standard or sample per well. The samples were diluted 1:10 in reagent diluent. The standard was first diluted to 4000 ng/ml, and then 2-fold serial diluted in 6 steps. Samples and the standard were added to the plate in duplicates, after which the plate was sealed and incubated for 2 hours at room temperature. 1-2 hours prior to use, the detection antibody was diluted to a working concentration of 200 ng/ml in reagent diluent with 2% normal goat serum. After the standard/sample incubation, the 5 washing steps were repeated and 100 µl detection antibody solution was added to each well. The plate was yet again sealed and incubated for 2 hours at room temperature. The 5 washing steps were performed once more, and 100 µl of Streptavidin-Horseradish peroxidase (HRP) working solution was added to each well. This working solution contained Streptavidin-HRP diluted 1:200 in reagent diluent. The enzyme solution was left to bind for 20 minutes at room temperature. Wells were washed 5 times, and 100 µl substrate solution was added to each well. The substrate solution was a 1:1 mixture of color reagent A (H_2O_2) and color reagent B (tetramethylbezidine). The plate was incubated with this mixture for 20 minutes at room temperature, after which 50 µl stop solution was added to each well. To ensure complete mixing, the plate was gently tapped. Finally, the optical density was determined using a Multiskan Ascent 354 plate reader (Labsystems) set to 414 nm (the reader did not have 450 nm as stated in the kit protocol). In addition, 550 nm readings were done, for manual wavelength correction. A standard curve was created by performing a four parameter logistic (4-PL) curve-fit using ELISA software at elisaanalysis.com. The data was further processed in Microsoft Excel.

2.8 Arachidonic and oleic acid release assay

The method for assessing the amount of AA and OA release used here is a radioactivity-based assay. The assay was started off by the cell experiment described in section 2.2.2, where the cells were labeled with radioactive AA and OA and subsequently treated with different TLR agonists. The AA/OA still left inside the cells' membrane and the AA/OA released in the cell culture supernatant can then be measured by determining the radioactivity of each component. The amount of AA in the supernatant will reflect the activity of PLA_2s releasing AA, such as GIVA $cPLA_2$, whereas the OA release will reflect the activity of other PLA_2s – mainly $iPLA_2$ and $sPLA_2$.

After the 48-well plates from section 2.2.2 had been incubated for the desired period, the cell supernatant from each well was transferred into eppendorf tubes. Detached cells were cleared away by centrifuging for 5 minutes at 13 200 rpm. 160 µl of the supernatant was transferred to eppendorf tubes containing 1 ml scintillation cocktail. 150 µl 1M NaOH was subsequently added to the wells now only containing cells. The cells were lysed by placing them on a hot plate at temp 1 for 7-8 minutes. Cell lysates were transferred to eppendorf tubes containing 1 ml scintillation cocktail. All the samples were mixed in the rack by placing another rack on top and turning this sandwich gently 3-4 times. Finally, the radioactivity of the samples was measured by liquid scintillation counting using a Tri-Carb 2900TR liquid scintillation analyzer (Packard). The resulting data of disintegrations per minute (DPM) were processed in Microsoft Excel.

2.9 Statistical analysis

For the qPCR experiments, randomisation tests performed by REST 2009 were used. Statistical analyses of ELISA and radioactivity assay results were performed using one-way ANOVA followed by Holm-Sidak multiple comparisons test in SigmaPlot 12.3. The p values < 0.05 were considered significant.

3. RESULTS

3.1 The SW982 cells express the RANKL, OPG and DKK1 genes

The RANKL, OPG and DKK1 system is central in bone metabolism, and is involved in RA pathogenesis. RANKL is required to activate bone-degrading osteoclasts, while OPG can block this activation by acting as a decoy receptor for RANKL [25]. DKK1 on the other hand, hinders differentiation of the bone-forming osteoblasts [26]. The SW982 cells are thought to originate from SFs, but there are no published data available regarding their RANKL, OPG and DKK1 gene expression. These genes were consequently investigated by qPCR analyses. It was found that synovial SW982 cells express RANKL, OPG and DKK1 (figure 3.1.1).

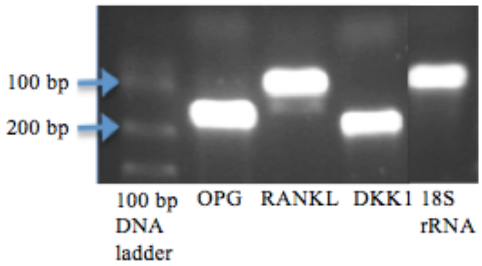

Figure 3.1.1 Gel electrophoresis showing the presence of OPG, RANKL and DKK1 gene expression in untreated SW982 cells. The presence of the reference gene, 18SrRNA, is shown as well.

The bands of the qPCR products from gel electrophoresis (figure 3.1.1) have the expected sizes for OPG (157 bp), RANKL (90 bp) and DKK1 (183 bp). This is also the case for 18SrRNA (100 bp), which was used as reference gene. The gel picture was only used as a qualitative verification. The gel bands of the qPCR products have reached saturation following 40 cycles of amplification. Therefore the intensity cannot be compared to determine expression levels.

The C_q values from qPCR suggest that the SW982 cells' expression level of OPG and DKK1 is similar, as both had average values around 23 (data not shown). RANKL is less expressed with an average C_q value of 29. The 18SrRNA gave C_q values around 5. Thus, as in most mammalian cells, 18SrRNA is highly expressed in the SW982 cells.

All weak shadows in the upper edge of the gel picture (figure 3.1.1) are residual primers. However, there is an additional weak band in the well of RANKL beneath the main band. This indicates that there is a nonspecific product present. This was also detected in the qPCR dissociation curve analysis, which shows the melting temperatures of the amplification products (figure 3.1.2). If the amplification had been specific, the melting curve would display only a single peak. This is not the case for the RANKL primer, because the melting curve has a small extra peak following the main peak. It was suggested by the supplier of the primer that this might be due to genomic DNA contamination in the samples, despite the fact that the Qiagen RNeasy Mini Kit is supposed to remove most of the genomic DNA. Therefore, DNase

digestion was performed in subsequent experiments. However, the RANKL qPCR melting curves still had an extra peak and even higher C_q values. As a result, it was decided to discontinue the use of the RANKL primer.

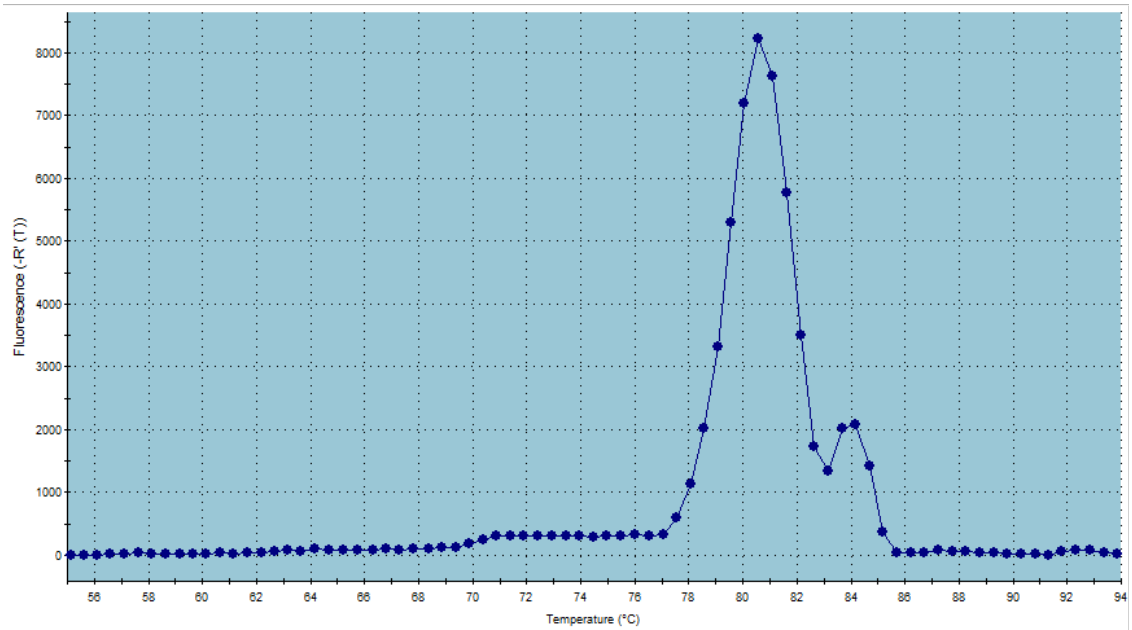

Figure 3.1.2 The dissociation curve of a qPCR product from using the RANKL primer displays an additional peak to the right of the main peak, indicating non-specific amplification.

The melting curves of OPG and DKK1 (not shown) gave only one single peak as expected of ready-made primers.

For all the primers used in this master's project the "no reverse transcriptase" control, for detection of genomic DNA contamination, gave C_q values that were 5 or more cycles higher than the genes of interest. This means that any DNA contamination will not affect gene expression analysis [80]. The "no template" control, which functions to reveal any nonspecific binding of the fluorescence binding dye SYBR Green I, gave C_q values above the detection limit (cycle 35) or no C_q value at all, for all the primers used. This indicates that minimal/no primer dimers or external DNA contamination were present in the qPCRs. Moreover, the amplification efficiency, accounted for in the expression analysis by the REST 2009 software, were 80% or higher for all primers.

To summarize, the SW982 cells express the OPG and DKK1 genes and may thus be capable of producing the corresponding proteins. Furthermore, the cells may express the RANKL gene, but this will need further characterization due to the possibility of non-specific detection.

3.2 The SW982 cells express the TLR1-TLR7 genes

The SFs are considered to be one of the dominant effector cells in RA, and they express certain TLRs. These receptors recognize different microbes, but can also bind endogenous molecules present in inflamed joints. TLRs are suggested to be some of the factors causing the perpetuation of the inflammation in RA [9]. With the goal of characterizing the expression of TLRs in the synovial SW982 cells, gene analysis by qPCR was performed. The SW982 cells were found to express all the TLRs in question: TLR1-TLR7 (figure 3.2.1).

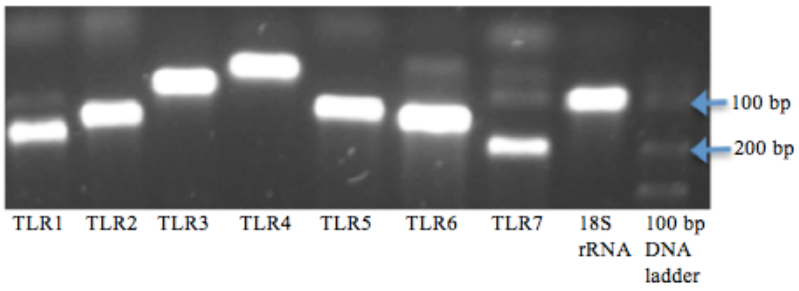

Figure 3.2.1 Gel electrophoresis showing the presence of TLR1-TLR7 gene expression in untreated SW982 cells. The presence of the reference gene, 18SrRNA, is shown as well.

The clear gel electrophoresis bands (figure 3.2.1) of TLR1 (189 bp), TLR2 (154 bp), TLR3 (97 bp), TLR4 (68 bp), TLR5 (136 bp), TLR6 (151 bp) and TLR7 (195 bp) have sizes as expected from the information provided by the primer supplier. The same goes for the reference 18SrRNA (100 bp). Again, the gel picture was only used as a qualitative verification.

The qPCR C_q values indicate that TLR2 is the TLR with the highest expression in the SW982 cells, with values of 21-22. TLR3, TLR4, and TLR6 follow behind with C_q values around 23-24, 25-26 and 25 respectively. TLR1, TLR5 and TLR7 have C_q values around 28-29, 29-30 and 30-31 respectively, and these are consequently the lowest expressed TLRs.

All weak shadows in the upper edge of the gel picture (figure 3.2.1) are residual primers. Additional weak bands can be seen above the main bands in the wells with TLR1, TLR6 and TLR7. The melting curve analysis resulted in one smooth peak for both TLR1 and TLR6 (not shown), but for TLR7 a small peak prior to the main peak was observed (figure 3.2.2). Such small, early peaks are often due to primer dimers, but this is not likely to be the cause here since the bands in the gel are not less than 50 base pairs. The explanation may be that the TLR7 primer is nonspecific, which can also be the case for the TLR1 and TLR6 primers even though nothing is abnormal with their melting curves.

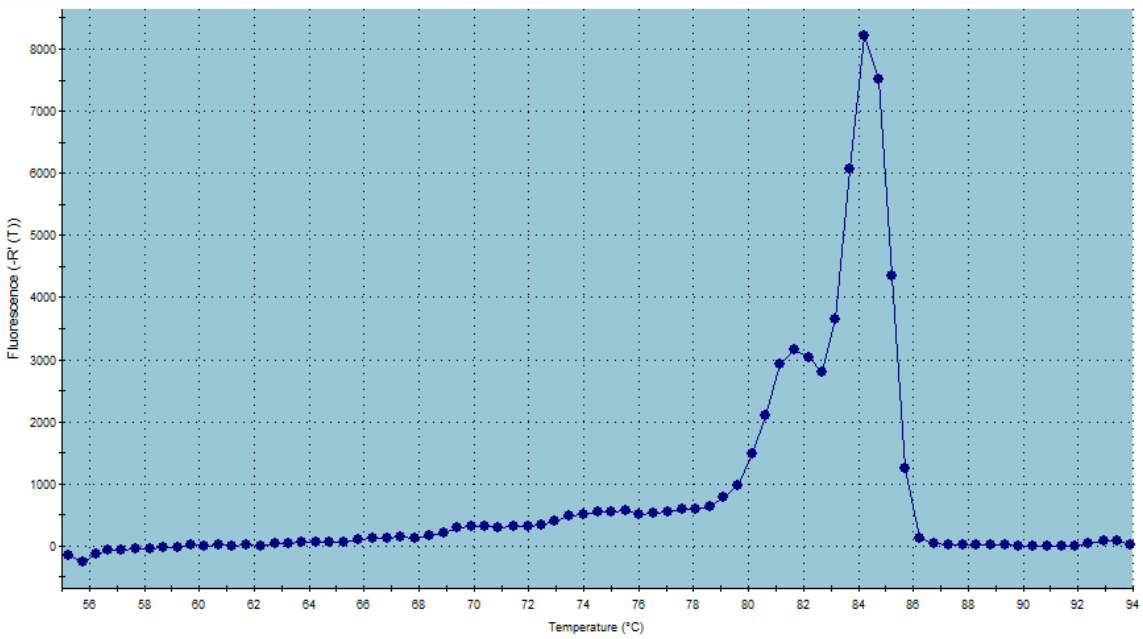

Figure 3.2.2 Dissociation curve of a qPCR product from using the TLR7 primer displays an additional peak to the left of the main peak, indicating primer dimers or non-specific amplification.

In summary, the SW982 cells express the TLR2, TLR3, TLR4, TLR5 genes, and probably TLR1, TLR6 and TLR7 too. There are indications that primers for the latter genes are nonspecific. Like the RANKL primer, these primers were not used any further.

3.3 TLR agonists increase OPG and DKK1 gene expression

Following the discovery that the SW982 cells express TLR1-TLR7, it was of interest to examine if TLR signaling affects the RANKL, OPG and DKK1 system in these cells. Because the RANKL primer was discarded for further use, only the OPG and DKK1 gene expression following TLR agonist stimulation were investigated. Gene analyses were done by qPCR.

Initial experiments were performed by stimulating the SW982 cells with the TLR2/6 agonist FSL-1, the TLR1/2 agonist P3C, the TLR3 agonist PIC, and the TLR4 agonist LPS, for 6 and 24 hours. This was in order to examine potential effects on the OPG and DKK1 gene expression levels, and whether these changed over time. Stimulation with the pro-inflammatory cytokine, IL-1β, was used as a positive control, because the signaling pathways activated by TLRs and IL-1β have many similarities [81]. This is due to the fact that the type I IL-1 receptor and the TLRs exhibit high homology in their cytosolic domain, which is for that exact reason called Toll/IL-1 receptor (TIR) domain. Moreover, IL-1β is known to induce gene expression of the characterized expressed genes in the SW982 cells [62].

The LPS batch seemed to be defect as it failed to evoke any transcriptional responses in the initial experiments performed. Even with ultrasonication, LPS stimulation may be hard to obtain [82]. Consequently, LPS treatment was discontinued in further experiments.

In all the quantitative gene expression analyses performed in this master's project, the expression of the gene of interest for the control (abbreviated by ctrl) is set to 1 and the expression of the treated samples is compared relative to this. This is referred to as fold change. Moreover, all figures presenting gene expression results show one representative experiment, unless otherwise is stated. The statistical significance shown in the figures is based on several biological replicas. The mean fold changes and the corresponding standard error of means (SEMs) for all replicas performed are presented in the appendix.

FSL-1, P3C and IL-1β all gave a significant increase in OPG mRNA level compared to the untreated control after 6 hours stimulation of SW982 cells (figure 3.3.1 A). The FSL-1 and IL-1β resulted in the highest OPG increase. This was somewhat higher than the increase by P3C. Furthermore, the induction of OPG by PIC after 6 hours was not significantly different from the control (figure 3.3.1 A). After 24 hours stimulation, the OPG mRNA expression seems to be reduced to levels closer to the control, as none of the TLR agonists and neither IL-1β gave a significant OPG increase (figure 3.3.1 B).

The expression of DKK1 after 6 hours stimulation showed similar trends as for OPG, with FSL-1 and IL-1β resulting in higher DKK1 mRNA increase than P3C and PIC (figure 3.3.1 C). However, the DKK1 up-regulation was smaller than seen for OPG, and none of them were significant. After 24 hours stimulation, the DKK1 fold changes were more uniform for all TLR agonists, but still not significantly different from the control (figure 3.3.1 D). Fold changes and SEMs from the two biological replicas, which the significance in this representative experiment is based on, are shown in appendix A.

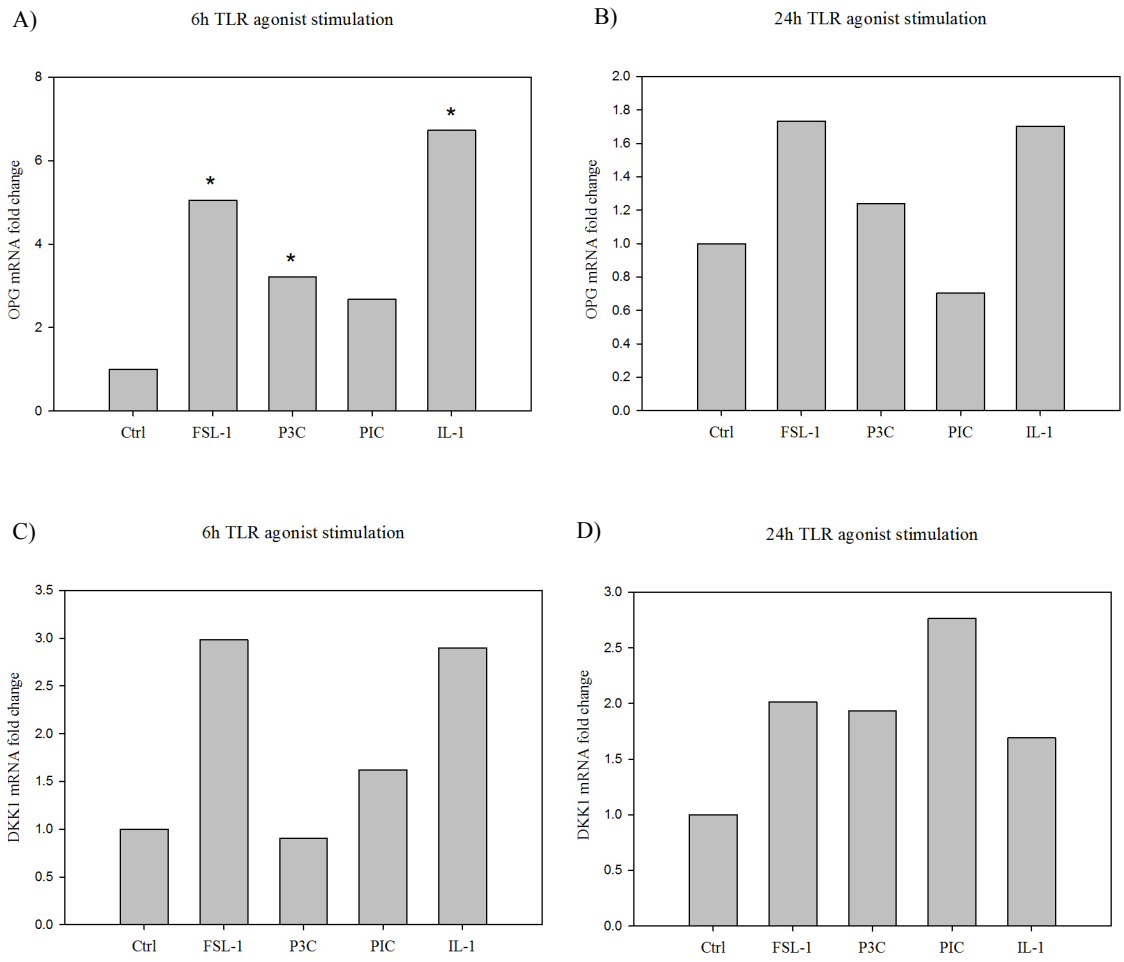

Figure 3.3.1 Relative OPG and DKK1 gene expressions obtained from REST 2009 using qPCR data from one representative experiment with SW982 cells. The asterisk indicates significant difference from control ($p < 0.05$). Statistical significance was calculated from the total number of biological replicas ($n = 2$). A) and B) show the OPG gene expression ratios after 6 and 24 hours of stimulation with the TLR2/6 agonist FSL-1, the TLR1/2 agonist P3C, and the TLR3 agonist PIC, plus the cytokine IL-1β. C) and D) show the DKK1 gene expression ratios after 6 and 24 hours of stimulation with the same TLR agonists and cytokine as in A) and B).

From the results of the 6 and 24 hours experiments, it was presumed that a short stimulation period for the TLR agonists were better for detecting an increase in OPG and DKK1 mRNA levels. The next goal was to more exactly find the optimal duration of stimulation. To achieve this, experiments with 3, 6, 9 and 12 hours stimulation periods of the SW982 cells were performed. All these stimulation periods resulted in increased OPG mRNA expression (figure 3.3.2 A). FSL-1 and IL-1β were still the strongest inducers of OPG expression, and the OPG levels tended to be highest after 3 and 6 hours stimulation. However, 9 hours stimulation was the only time period that gave a significant OPG increase for all the TLR agonists. Again, the DKK1 gene was less responsive and its mRNA levels did not seem to increase until after 6 hours of stimulation (figure 3.3.2 A). Also for DKK1, 9 hours stimulation was the time period giving significant up-regulation for all the TLR agonists. The time curves (figure 3.3.2) are based on two biological replicas, and the mean fold changes and the SEMs are shown in appendix B.

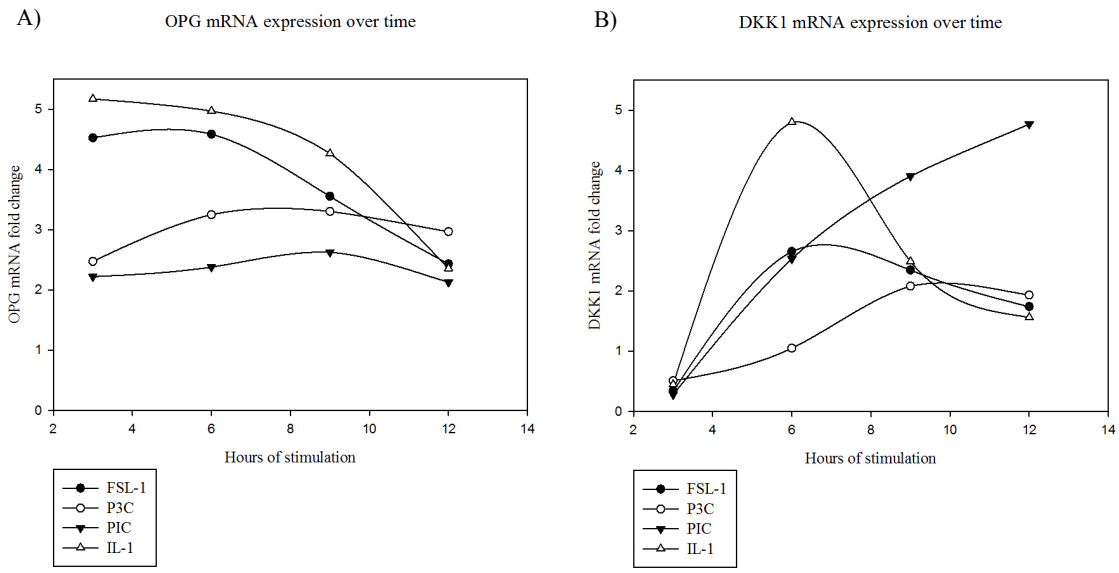

Figure 3.3.2 Time curves of mean relative gene expressions obtained from REST 2009 using qPCR data from experiments (n = 2 for all TLR agonists, n = 1 for IL-1β) with SW982 cells. Error bars and significance are not shown (see appendix B). A) The OPG mRNA fold changes after 3, 6, 9 and 12 hours of stimulation with the TLR2/6 agonist FSL-1, the TLR1/2 agonist P3C and the TLR3 agonist PIC, plus the cytokine IL-1β. B) The DKK1 mRNA fold changes after 3, 6, 9 and 12 hours of stimulation with the same TLR agonists and cytokine as in A).

In summary, treatment of the SW982 cells with FSL-1, P3C, PIC or IL-1β, increases the OPG and DKK1 gene expression. The stimulation time that gave a significant increase in the mRNA levels of these two genes, was found to be 9 hours. Moreover, the fact that the cells respond to these TLR agonists confirms that they express the corresponding TLRs and that they are functional.

3.4 OPG protein secretion is enhanced by TLR agonists increase

As a consequence of the novel discovery that the TLR agonists induce OPG and DKK1 gene expression in the SW982 cells, it was decided to proceed with analysis at protein level. However, due to limited time and because OPG seemed to be slightly more responsive than DKK1, protein analyses were only performed for OPG. The analyses were performed using SW982 cell culture supernatants, from the same experiments as from where the cell material was analyzed at gene level, by sandwich ELISA.

OPG protein level after 12 hours stimulation with the TLR agonists was compared to the 9 hours stimulation samples in initial analyses. The OPG protein concentration obtained from the 9 and 12 hours experiments did not differ much and they both showed the same trends. Moreover, both time periods resulted in similar OPG protein up-regulation relative to their control (data not shown).

In the subsequent ELISAs, only supernatants from 9 hours experiments were further analyzed. FSL-1, P3C, PIC, and IL-1β all resulted in significant increase in OPG protein levels (figure 3.4.1). FSL-1 and IL-1β induced the highest OPG protein production, with a mean concentration of 17.5 ± 3.2 and 17.4 ± 2.7 ng/ml respectively. P3C and PIC stimulation led to significantly less induction, with mean concentrations of 10.7 ± 1.5 and 9.2 ± 2.3 ng/ml respectively. However, there was no significant difference between these two. The mean OPG concentration of the control was 4.3 ± 1.2 ng/ml.

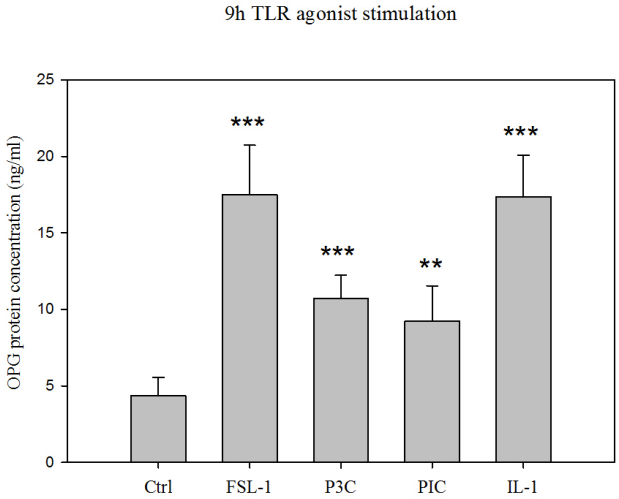

Figure 3.4.1 The mean concentration of OPG (ng/ml) in SW982 cell culture supernatants, measured by ELISA, after 9 hours stimulation with the TLR2/6 agonist FSL-1, the TLR1/2 agonist P3C, the TLR3 agonist PIC, or the cytokine IL-1β. The asterisks indicate significant difference from control (* p = 0.01 - 0.05, ** p = 0.001 - 0.01, *** p < 0.001), and the error bars denote ± SD (n = 7 for FSL-1 and P3C, while n = 5 for PIC and IL-1).

The trend of FSL-1 and IL-1β giving the highest induction after 9 hours is the same trend as was seen for the OPG mRNA levels after 6 hours stimulation (figure 3.3.1 A). This is as anticipated, because there is always a delay between gene expression and protein expression.

3.5 TLR agonist-induced OPG expression involves COX activity

Having found that SW982 cells increase their expression of OPG in response to certain TLR agonists and IL-1β, both on gene and protein level, it was of interest to investigate whether the $cPLA_2$ enzyme is an upstream regulator of the OPG gene. The $cPLA_2$ is considered to be the most central enzyme for formation of AA, which is converted into prostanoid precursors by COX-1 and COX-2. These precursors are further converted into inter alia PGs, which are found to be more prominent in bone tissue suffering from inflammation [40]. Finding out if $cPLA_2$ is involved in the TLR agonist-induced expression of OPG, is a part of the attempt to clarify the TLR signaling events in the SW982 cell model for RA. It is of interest to see whether targeting the pathway responsible for AA and subsequently PG formation has any effect on OPG – the molecule capable of blocking differentiation of bone-degrading osteoclasts. With the aim of clarifying this, the SW982 cells were treated with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or a COX inhibitor, Indomethacin, each in combination with the different TLR agonists, for 9 hours. As before, the gene analysis was done by qPCR.

Due to time limitation, the gene analysis was only performed for FSL-1 and P3C. PIC was not included because it seemed to affect the cells negatively, as will be further described in the discussion chapter. Both FSL-1 and P3C showed similar levels of increase in the OPG mRNA expression, with fold induction values somewhat above 3 (figure 3.5.1). Neither of the $cPLA_2$ inhibitors, nor the COX inhibitor, gave any significant effect on the FSL-1-induced increase of mRNA OPG (3.5.1 A). In the case of P3C and inhibitors, however, there seemed to be a more distinct trend of reduction in the OPG mRNA levels compared to the TLR agonist alone, although only Indomethacin gave a significant inhibition (figure 3.5.1 B). Fold changes and SEMs from the five biological replicas, which the significance in this representative experiment is based on, are shown in appendix C.

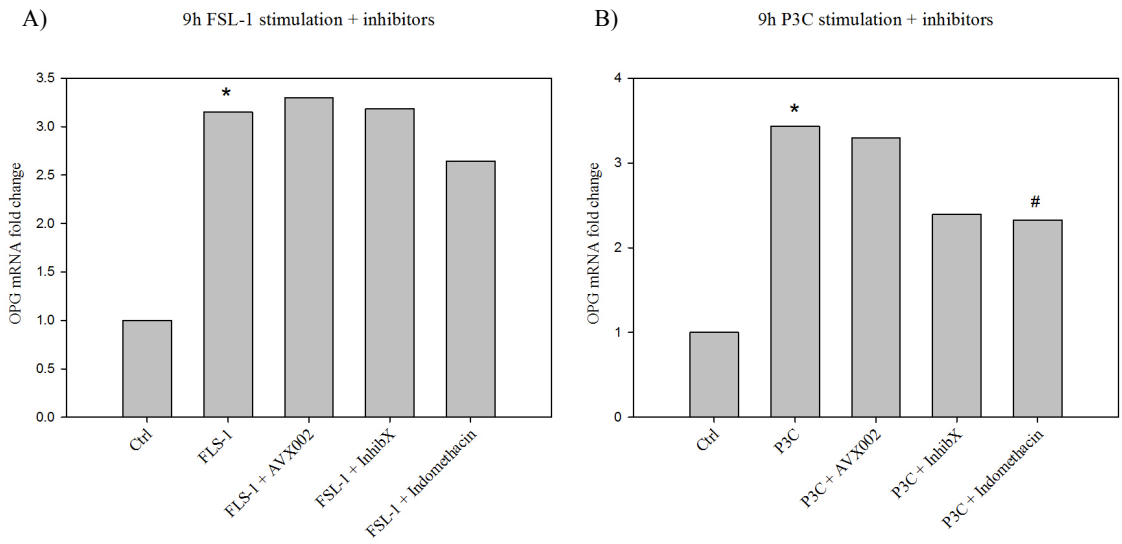

Figure 3.5.1 Relative OPG gene expressions obtained from REST 2009 using qPCR data from a representative experiment with SW982 cells. The asterisk indicates significant difference from control ($p < 0.05$), and the number sign denotes significant difference from the TLR agonist alone ($p < 0.05$). Statistical significance was calculated from the total number of biological replicas ($n = 5$). A) The OPG gene expression ratios after 9 hours of stimulation with the TLR2/6 agonist FSL-1 in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin. B) The OPG gene expression ratios after 9 hours of stimulation with the TLR1/2 agonist P3C in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin.

Subsequently, the OPG protein concentration in cell culture supernatants from 9 hours treatment with the $cPLA_2$ inhibitors and the COX inhibitor were analyzed by sandwich ELISA, to see whether the protein levels corresponded with the results obtained at mRNA level. These protein analyses also included PIC and the pro-inflammatory cytokine IL-1β, in addition to FSL-1 and P3C. FSL-1, P3C and IL-1β alone all gave significant increase in the OPG protein level, but not PIC (figure 3.5.2). Still, FSL-1 and IL-1β results in the highest and similar induction. None of the inhibitors gave a significant reduction in the FSL-1-induced OPG protein increase (figure 3.5.2 A), which is the same as detected on the mRNA level. Nor was there any significant reductions found for the P3C-induced OPG protein increase with the different inhibitors (figure 3.5.2 B). Thus, the significant reduction in the P3C-induced OPG response by Indomethacin found at gene level was not detected at protein level. Furthermore, no significant inhibition of the PIC- and IL-1β-induced OPG protein increase was found (figure 3.5.2 C and D).

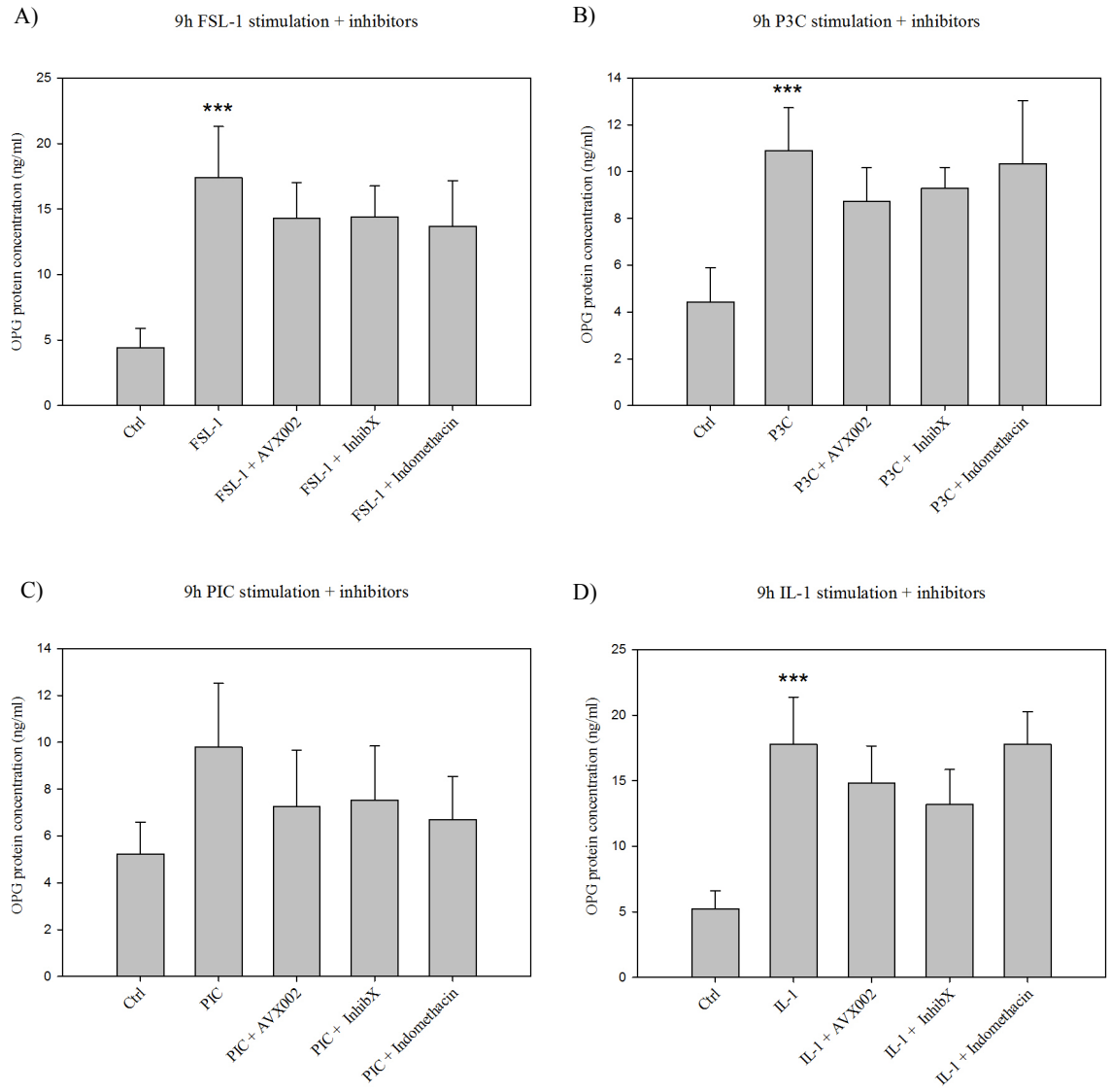

Figure 3.5.2 The mean concentration of OPG (ng/ml) in SW982 cell culture supernatants measured by ELISA. The asterisks indicate significant difference from control (*** $p < 0.001$). If present, a number sign would denote significant difference from the TLR agonist alone ($p < 0.05$). The error bars indicate ± SD ($n = 5$ for FSL-1 and P3C, $n = 3$ for PIC and IL-1β). A) 9 hours stimulation with the TLR2/6 agonist FSL-1 in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin. B) 9 hours stimulation with the TLR1/2 agonist P3C in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin. C) 9 hours stimulation with the TLR3 agonist PIC in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin. D) 9 hours stimulation with the cytokine IL-1β in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin.

In summary, OPG expression was found to be induced by the TLR agonists FSL-1 and P3C, both on mRNA and protein level. The PG pathway does not seem to be involved in the FSL-1-mediated OPG expression, as neither $cPLA_2$ inhibitors, nor a COX inhibitor gave significant changes. This is valid for both OPG mRNA and protein. However, inhibitory tendencies, although not significant, were seen at protein level. The P3C-mediated OPG expression, on the other hand, might involve the PG pathway, because the COX inhibitor showed significant reduction of OPG mRNA. However, this was not significantly reflected at protein level. Furthermore, the TLR agonist PIC and the cytokine IL-1β resulted in enhanced levels of OPG protein. As stated earlier, these two treatments were not analyzed at gene level.

3.6 The $cPLA_2$ and COX enzymes are involved in TLR agonist-induced DKK1 expression

DKK1 is an inhibitor of the WNT pathway, which is important for differentiation and function of the bone-forming osteoblasts [26]. Although a slightly lower response was detected for DKK1 mRNA in the initial TLR agonist experiments, the DKK1 gene expression was investigated further for 9 hours stimulation with TLR agonists combined with either of the $cPLA_2$ inhibitors, AVX002 and InhibX, or the COX inhibitor, Indomethacin. To further elucidate the TLR signaling and find out whether there is a connection to key components in bone remodeling, the involvement of the PG pathway in the TLR agonist-induced DKK1 response in SW982 cells was examined.

As for OPG, the gene analysis was only performed for the FSL-1 and the P3C. The DKK1 fold change for FSL-1 was slightly above 2, which was not significantly different from the control. Regarding the inhibitors, only Indomethacin gave a significant inhibition (figure 3.6.1 A). In the initial 6 hours experiments, the DKK1 mRNA expression was higher after FSL-1 treatment than for P3C. However, following 9 hours stimulation P3C seems to be a better inducer than FSL-1, increasing DKK1 gene expression nearly a 3-fold (figure 3.6.1 B). Both $cPLA_2$ inhibitors and the COX inhibitor significantly reduced the P3C-induced increase in DKK1 mRNA levels. The AVX002 and InhibX gave similar levels of inhibition, while Indomethacin seemed to be even more effective. All P3C and inhibitor treatments resulted in DKK1 fold changes that were close to the control. Fold changes and SEMs from the five biological replicas, which the significance in this representative experiment is based on, are shown in appendix D.

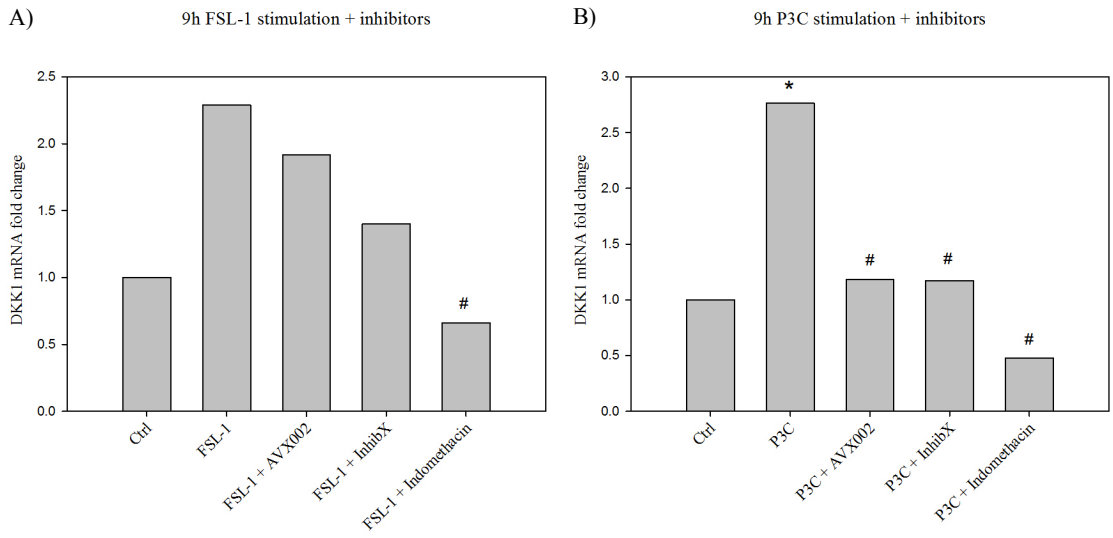

Figure 3.6.1 Relative DKK1 gene expressions obtained from REST 2009 using qPCR data from one representative experiment with SW982 cells. The asterisk indicates significant difference from control ($p < 0.05$), and the number sign denotes significant difference from the TLR agonist alone ($p < 0.05$). Statistical significance was calculated from the total number of biological replicas ($n = 5$). A) The DKK1 gene expression ratios after 9 hours of stimulation with the TLR2/6 agonist FSL-1 in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin. B) The DKK1 gene expression ratios after 9 hours of stimulation with the TLR1/2 agonist P3C in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin.

To summarize, the TLR agonists, FSL-1 and P3C, resulted in up-regulation of the DKK1 gene, although this was non-significant for FSL-1. Each agonist treatment showed inhibitory tendencies for both $cPLA_2$ inhibitors and the COX inhibitor. However, the down-regulation of DKK1 by all inhibitors was only significant for P3C. The COX inhibitor resulted in significant inhibition for FSL-1. These results suggest that the PG pathway is involved in P3C-mediated DKK1 expression. The involvement of this pathway in FSL-1-mediated DKK1 expression is less clear, but still possible.

3.7 TLR agonist-induced IL-6 expression involves $cPLA_2$ and COX enzymes

The SW982 cells are known to express the pro-inflammatory cytokine IL-6, and the expression is highly enhanced by IL-1β [62]. IL-6 is one of the factors responsible for perpetuating the synovitis in RA by promoting B cell antibody production and activation of T cells, macrophages and osteoclasts [1]. In the TLR agonist experiments, the target genes, IL-6 and COX-2 (see next section for COX-2), were used as positive controls to ensure that the induction had been successful – although the result in itself is interesting for mapping TLR signaling events in the SW982 cells.

Both FSL-1 and P3C were found to be strong inducers of IL-6 mRNA expression in the SW982 cells, with fold change values of 45 and 31 respectively (figure 3.7.1) – i.e. much higher induction than found for OPG and DKK1. The $cPLA_2$ inhibitors, AVX002 and InhibX, did not give any significant effect on the FSL-1-induced IL-6 expression, but the COX inhibitor, Indomethacin, gave a significant reduction (figure 3.7.1 A). For the P3C-induced IL-6 expression, all the inhibitors resulted in significant inhibition (figure 3.7.1 B). Fold changes and SEMs from the five biological replicas, which the significance in this representative experiment is based on, are shown in appendix E.

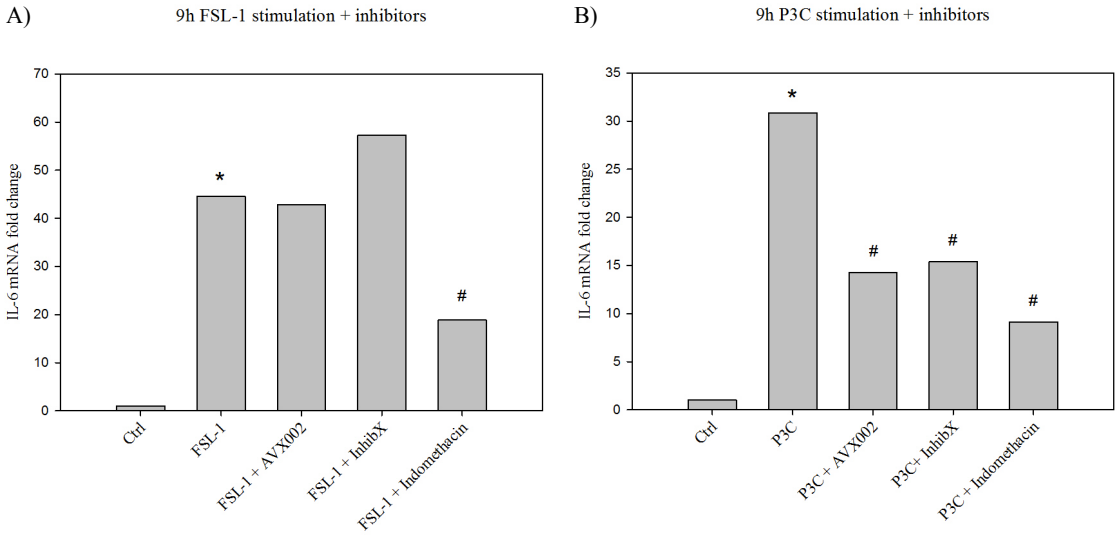

Figure 3.7.1 Relative IL-6 gene expressions obtained from REST 2009 using qPCR data from one representative experiment with SW982 cells. The asterisk indicates significant difference from control ($p < 0.05$), and the number sign denotes significant difference from the TLR agonist alone ($p < 0.05$). Statistical significance was calculated from the total number of biological replicas ($n = 5$). A) The IL-6 gene expression ratios after 9 hours of stimulation with the TLR2/6 agonist FSL-1 in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor. Indomethacin. B) The IL-6 gene expression ratios after 9 hours of stimulation with the TLR1/2 agonist P3C in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin.

In conclusion, both TLR agonists, FSL-1 and P3C, resulted in extensive induction of the IL-6 gene, implying that the TLR agonist stimulation was successful. Only the COX inhibitor had significant effect on FSL-1-mediated IL-6 expression. For P3C, however, both $cPLA_2$ inhibitors and the COX inhibitor reduced the IL-6 induction significantly. This indicates that the PG pathway may contribute to IL-6 increase following FSL-1 stimulation, and the pathway is indeed involved in P3C-mediated IL-6 expression.

3.8 TLR agonist-induced COX-2 is affected by $cPLA_2$ and COX inhibitors

Like IL-6, the COX-2 gene is among the genes whose expression has been characterized in the synovial SW982 cells, and it is known to be strongly induced by IL-1β [62]. As stated earlier, the COX-1 and COX-2 enzymes are responsible for converting AA into precursors of inter alia PGs, which are prominent in RA. COX-1 is constitutive and is accountable for the basal levels of PGs, while COX-2 is the inducible isoform [83].

Both FSL-1 and P3C were found to highly induce COX-2 mRNA in the SW982 cells, with fold changes of 24 and 13 respectively (figure 3.8.1) – markedly higher than the OPG and DKK1 induction, but still not as much as the IL-6 induction. As for IL-6, FSL-1 was the most potent inducer. Only the COX inhibitor Indomethacin gave a significant reduction of the FSL-1-mediated COX-2 induction (figure 3.8.1 A), which was also the case for IL-6. The P3C-mediated COX-2 expression, on the other hand, was significantly inhibited by the two $cPLA_2$ inhibitors, AVX002 and InhibX, and the COX inhibitor, Indomethacin (figure 3.8.1 B). Fold changes and SEMs from the five biological replicas, which the significance in this representative experiment is based on, are shown in appendix F.

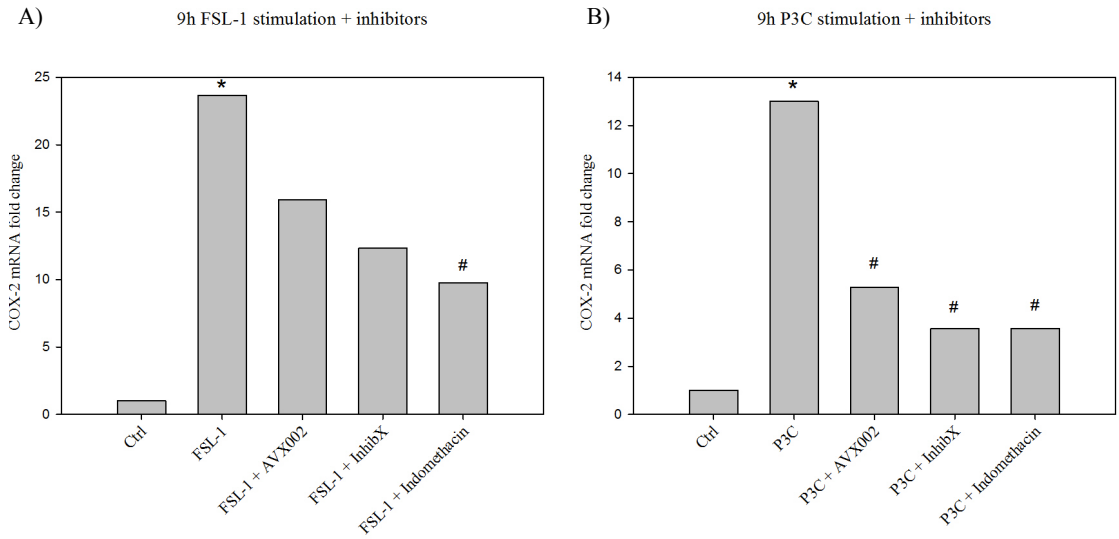

Figure 3.8.1 Relative COX-2 gene expressions obtained from REST 2009 using qPCR data from one representative experiment with SW982 cells. The asterisk indicates significant difference from control ($p < 0.05$), and the number sign denotes significant difference from the TLR agonist alone ($p < 0.05$). Statistical significance was calculated from the total number of biological replicas ($n = 5$). A) The COX-2 gene expression ratios after 9 hours of stimulation with the TLR2/6 agonist FSL-1 in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin. B) The COX-2 gene expression ratios after 9 hours of stimulation with the TLR1/2 agonist P3C in combination with either of the $cPLA_2$ inhibitors, AVX002 or InhibX, or the COX inhibitor, Indomethacin.

In conclusion, both TLR agonists, FSL-1 and P3C, resulted in a high up-regulation of the COX-2 gene, providing yet another proof that the TLR agonist stimulation was successful. Although all inhibitors used showed inhibitory tendencies, only the COX inhibitor had significant effect on FSL-1-mediated COX-2 expression. As for IL-6, both $cPLA_2$ inhibitors and the COX inhibitor reduced the P3C-mediated COX-2 expression significantly. Hence, the PG pathway contributes to the P3C-mediated COX-2 expression, and possibly also to the FSL-1-mediated.

3.9 AA release increases in response to TLR agonists

The aforementioned PGs, which are found to be elevated in RA, belong to the eicosanoid class of lipid mediators. Eicosanoids can be derived from AA, which is released from membrane phospholipids by PLA_2s [39]. There are four different groups of PLA_2 enzymes: $cPLA_2$, $sPLA_2$, $iPLA_2$, and $LpPLA_2$. All these have several subgroups [52]. The $cPLA_2$ and the $sPLA_2$ groups are considered the most important for PG formation, and GIVA $cPLA_2$ is believed to be the major enzyme for AA release [40]. In parallel to AA release, certain cell models have been found to release OA, due to the actions of $sPLA_2$ and $iPLA_2$ [42, 43].

To establish whether there is a connection between TLR signaling and the PG pathway in the human synovial SW982 cells, the activity of PLA_2 enzymes following TLR activation were investigated. This was done by determining the cells' AA release after TLR agonist stimulation by a radioactivity assay. In addition, OA release was investigated. IL-1β was used as a positive control, because this cytokine is known to stimulate AA release in human SFs [84]. Different TLR agonist concentrations were tested over the same time periods as for the gene expression analysis, namely 3, 6, 9, 12 and 24 hours. The results are given as fold change relative to the control, which are set to 1.

The FSL-1, P3C and PIC all resulted in increased levels of AA release compared to untreated cells (figure 3.9.1). For FSL-1, the AA release was significantly increased after 6 hours for both the concentrations used, 100 ng/ml and 50 ng/ml (3.9.1 A). The FSL-1-mediated AA release peaked at a fold change of 4.4 after 9 hours with 100 ng/ml, but there was no significant difference between the two FSL-1 concentrations. P3C significantly increased the AA release after 9 hours stimulation, which also was its maximum with a fold change of 3 (figure 3.9.1 B). However, this was only significant for 300 ng/ml, which generally resulted in higher release. The AA release following PIC stimulation was significantly increased already after 6 hours (figure 3.9.1 C). For all time periods, there was no significant difference between 5 µg/ml and 1 µg/ml PIC. PIC-mediated AA release peaked after 12 hours with a fold change of 2.9. As expected, the positive control IL-1β resulted in elevated AA levels (figure 3.9.1 D). The IL-1β time curve peaks at 9 hours with a fold change value of 4.5. This response is very similar to that of FSL-1. The time curves (figure 3.9.1) are based on three biological replicas, and the mean fold changes and the standard deviations (SDs) are shown in appendix G.

The OA release, on the other hand, generally showed minimal, non-significant changes in response to the TLR agonists used here (data not shown).

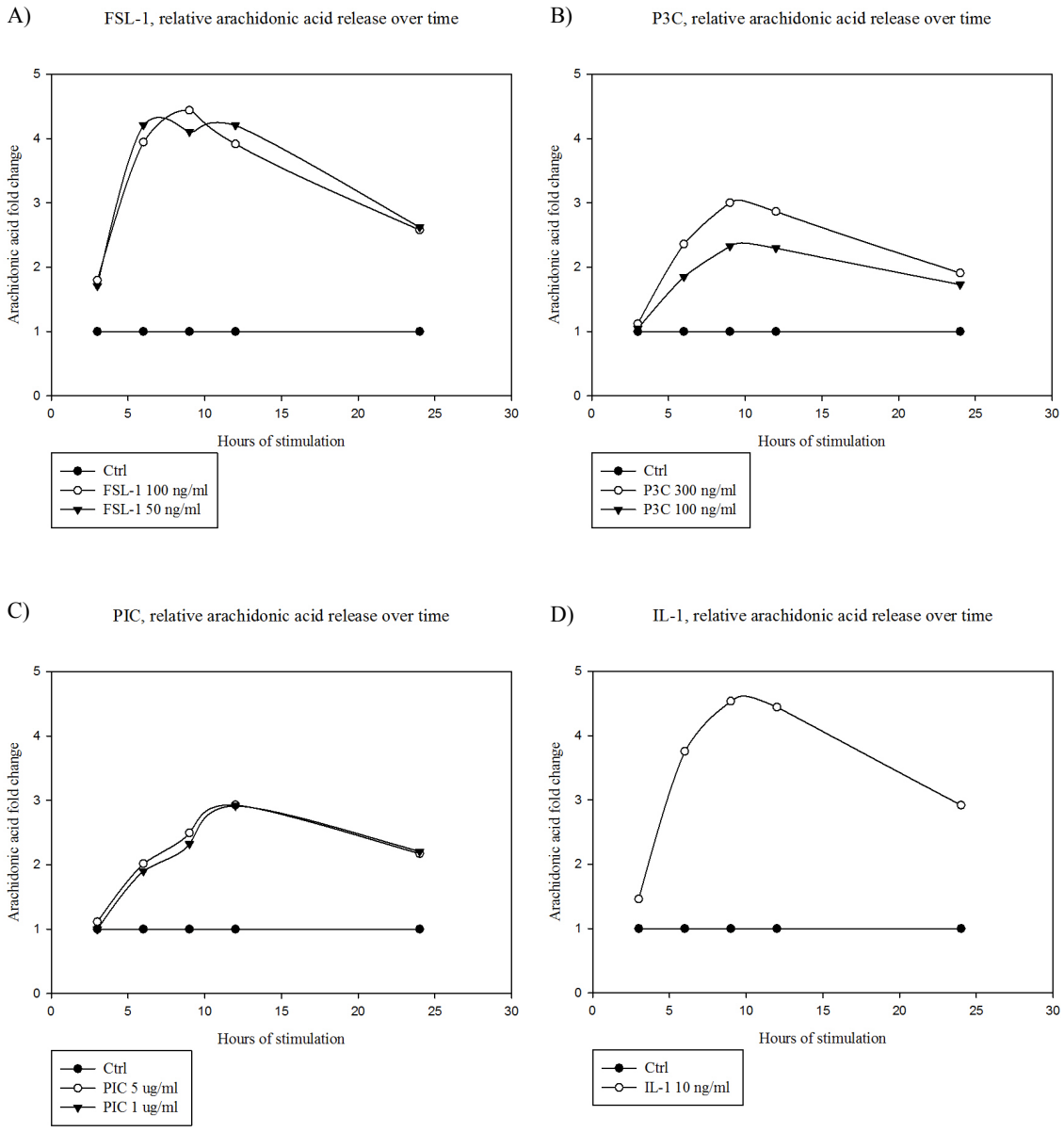

Figure 3.9.1 Time curves of mean fold change (n = 3) of AA release in culture medium after TLR agonist stimulation of SW982 cells. The curves were generated from data obtained by radioactivity assays. Error bars and significance are not shown (see appendix G). A) Relative AA release after stimulation with the TLR2/6 agonist FSL-1. B) Relative AA release after stimulation with the TLR1/2 agonist P3C. C) Relative AA release after stimulation with the TLR3 agonist PIC. D) Relative AA release after stimulation with the cytokine IL-1β.

To summarize, FSL-1, P3C, and IL-1β treatments led to increased AA release by the SW982 cells, and this peaked after 9 hours stimulation. There was little difference between 100 and 50 ng/ml FSL-1. The P3C response, on the other hand, was higher with the 300 ng/ml concentration, than with 100 ng/ml. PIC enhanced the AA release, but had its peak after 12 hours. However, this was not significantly different from the increase seen after 9 hours. There was no noticeable difference between 5 µg/ml and 1 µg/ml PIC. Of the TLR agonists used here, FSL-1 was the one resulting in the highest AA release, which was comparable to that of IL-1β.

3.10 The GIVA $cPLA_2$ is the dominant PLA_2 responsible for the TLR agonist-induced AA release

Having discovered that all the TLR agonists in question resulted in a significant increase in AA release from the SW982 cells, the next goal was to reveal which PLA_2 enzymes were responsible. As stated in the last section, the FSL-1- and P3C-mediated AA release peaked after 9 hours, and PIC also resulted in significant AA release increase with this stimulation time. Therefore, 9 hours were chosen as the stimulation time for the next experiments. To find the responsible PLA_2 enzymes, the same radioactivity assay used in the last section was performed. However, before stimulating with TLR agonists, the SW982 cells were pre-treated with different $cPLA_2$, $sPLA_2$, and $iPLA_2$ inhibitors for 2 hours. The inhibitors used and their details are listed in table 2.1 under "Materials and methods". By inhibiting specific PLA_2 enzymes, it is possible to suggest which of them that may contribute to the TLR-mediated AA release.

Both GIVA $cPLA_2$ inhibitors, AVX002 and CAY10502, were found to significantly reduce the FSL-1-mediated AA release from the SW982 cells (figure 3.10.1 A). In fact, CAY10502 resulted in complete inhibition down to basal level of AA release, while AVX002 showed around 60% inhibition. Furthermore, none of the $sPLA_2$ inhibitors, CAY10590 and Varespladib, significantly affected the FSL-1-mediated AA release. The $iPLA_2$ inhibitor BEL, on the other hand, had an inhibitory effect that was similar to that of AVX002. MAFP, which inhibits both $iPLA_2$ and $cPLA_2$, resulted in nearly complete inhibition. In the case of the P3C-mediated AA release, the different PLA_2 inhibitors showed the same inhibition trends as for FSL-1 (figure 3.10.1 B). The inhibitors' effects on the PIC-mediated AA release turned out to have similar tendencies as for FSL-1 and P3C (figure 3.10.1 C). However, the inhibitory effects of the $cPLA_2$ inhibitor, AVX002, and the $iPLA_2$ inhibitor, BEL, were non-significant.

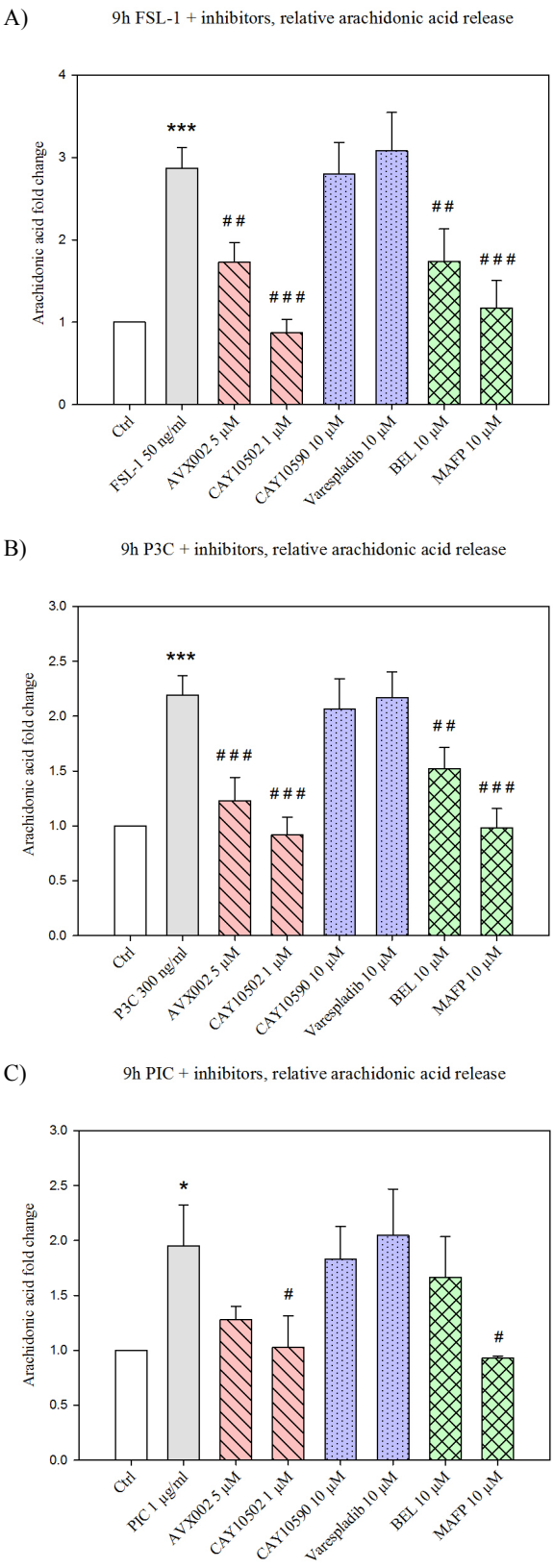

Figure 3.10.1 The mean fold change of AA release in culture medium after combination treatment with a TLR agonist and a PLA_2 inhibitor. The red bars are a TLR agonist + $cPLA_2$ inhibitors, the blue bars are a TLR agonist + $sPLA_2$ inhibitors, and the green bars are a TLR agonist + $iPLA_2$ inhibitors (MAFP inhibits both $iPLA_2$ and $cPLA_2$). The charts were generated from data obtained by radioactivity assays. The asterisks indicate significant difference from control (* p = 0.01 - 0.05, ** p = 0.001 - 0.01, *** p < 0.001), and the number signs indicate significant difference from the TLR agonist alone (# p = 0.01 - 0.05, # # p = 0.001 - 0.01, # # # p < 0.001). Error bars denote ± SD (n = 3) A) Relative AA release after stimulation with the TLR2/6 agonist FSL-1 and the different PLA_2 inhibitors. B) Relative AA release after stimulation with the TLR1/2 agonist P3C and the different PLA_2 inhibitors. C) Relative AA release after stimulation with the TLR3 agonist PIC and the different PLA_2 inhibitors.

In summary, the TLR agonists, FSL-1, P3C and PIC, all resulted in increased levels of AA release. GIVA $cPLA_2$ seems to be the dominant PLA_2 responsible for this. In addition, there are signs of possible $iPLA_2$ involvement.

3.11 TNF-α increases DKK1 gene expression

As mentioned in the first result section, RANKL, OPG and DKK1 are key components in bone remodeling. RANKL is a ligand essential to differentiation of the bone-degrading osteoclasts, OPG can hinder osteoclast activation by competitive binding of RANKL, and DKK1 is an inhibitor of the bone-forming pathway. The pro-inflammatory cytokine TNF-α is one of the factors believed to be a major contributor to RA pathogenesis, by e.g. induction of osteoclast formation [26].

A former master's student in the PLA_2 research group, Ahmed Siddik, investigated the effect of TNF-α on RANKL, OPG and DKK1 expression in the SW982 cells. By qPCR, it was found that TNF-α increased the expression of OPG and DKK1. The up-regulation of TNF-induced OPG and DKK1 was small, but significant. Regarding RANKL, the primers used then did not work well in qPCR and the results were inconclusive.

In this master's project, qPCR analyses of SW982 cells stimulated in 6, 12, 24 and 48 hours with TNF-α were performed to examine whether the RANKL, OPG and DKK1 mRNA expression changed over time. This was performed prior to discontinuing the use of the RANKL primer, and it was during these experiments the primer was found to be nonspecific, as described in section 3.1.

TNF-α gave no significant induction of RANKL for any of the stimulation periods (figure 3.11.1 A). This was also the case for OPG, whose expression showed even more statistical uncertainties (data not shown). DKK1 however, showed a significant 2.8-fold change after 6 hours TNF-α stimulation (figure 3.11.1 B). Fold changes and SEMs from the three biological replicas, which the significance in this representative experiment is based on, are shown in appendix H.

In the above-mentioned experiments, the $cPLA_2$ inhibitors AVX002 and InhibX were included in two different concentrations (1 and 5 μM) to examine whether potential TNF-α-induced RANKL, OPG or DKK1 expression involved the $cPLA_2$ enzyme and whether this was time and/or dose dependent. As stated above, neither RANKL nor OPG showed a significant increase after TNF-α treatment of the SW982 cells, while DKK1 gene expression did increase significantly after 6 hours. Furthermore, none of the $cPLA_2$ inhibitors were found to give significant effects for any of the genes (data not shown).

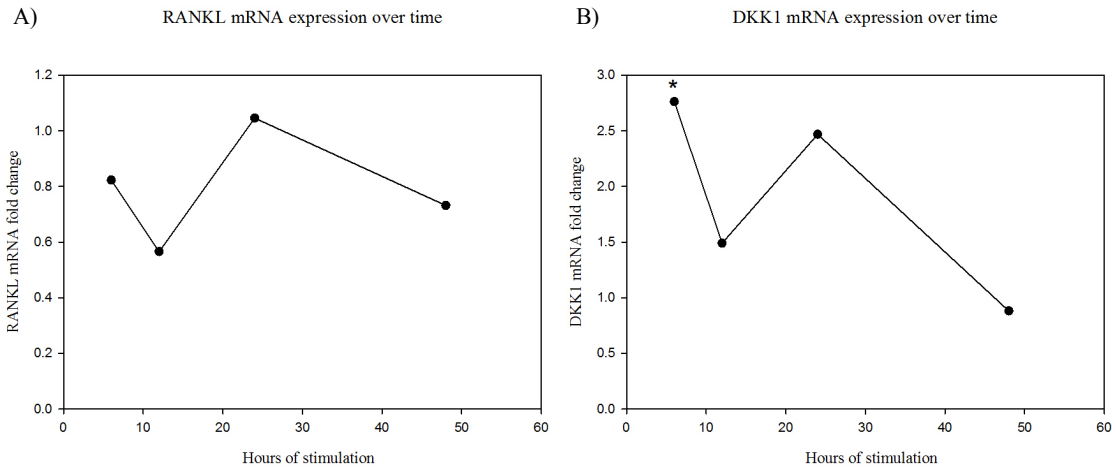

Figure 3.11.1 Time curves of relative gene expressions obtained from REST 2009 using qPCR data from one representative experiment with SW982 cells. The asterisk indicates significant difference from control ($p < 0.05$). Statistical significance was calculated from the total number of biological replicas (n = 3). A) The RANKL mRNA fold changes after 6, 12, 24 and 48 hours of TNF-α stimulation. B) The DKK1 mRNA fold changes after 6, 12, 24 and 48 hours of TNF-α stimulation.

RANKL and OPG gene expression analysis following TNF-α treatment were inconclusive, and the RANKL primer was suspected to be nonspecific. The DKK1 gene, however, was significantly up-regulated after 6 hours TNF-α stimulation. The combination treatment with a $cPLA_2$ inhibitor plus TNF-α showed no response in expression levels for any of the genes.

3.12 TLR2 and TLR3 gene expressions increase in response to TNF-α

SFs are known to express certain TLRs, and as shown in previous result sections, the SW982 cells express all TLRs from 1 to 7. TLRs are activated by molecular patterns on microbes and by endogenous molecules, such as those present in inflamed joints. This activation results in inter alia production of cytokines, such as IL-1, IL-6 and TNF-α [9].

6, 12, 24 and 48 hours TNF-α stimulation of the SW982 cells were performed followed by qPCR analyses, to investigate whether the TLRs expressed by these cells are affected by TNF-α and whether it would change over time. It was during these experiments there were found indications that TLR1, TLR6, and TLR7 primers may be nonspecific, as described in section 3.2.

TLR1 showed no distinct induction by TNF-α (figure 3.12.1 A). For all stimulation periods, the TLR1 fold change was not significantly different from the control. This was also the case for TLR4, TLR5, TLR6 and TLR7 (data not shown). TLR2 and TLR3, on the other hand, showed significant increase in mRNA levels after TNF-α stimulation. TLR2 was the one that resulted in the highest induction, with fold change values of 4.6 and 4.2 after 6 and 12 hours (figure 3.12.1 B). The expression seemed to decrease for longer stimulation periods. TLR3 fold change was 2.2 after 6 hours and 2.3 after 24 hours of TNF-α stimulation, and from there it decreased with time (figure 3.12.1 C). Fold changes and SEMs from the three biological replicas, which the significance in this representative experiment is based on, are shown in appendix I.

In addition, the time-lapse experiments included the $cPLA_2$ inhibitors, AVX002 and InhibX, in order to investigate if TNF-α-induced TLR expression involves the $cPLA_2$ enzyme. The inhibitors were used in two different concentrations (1 and 5 μM) to see if a potential response was time and/or dose dependent. However, the inhibitors showed no significant effect for any of the TLRs in question (data not shown).

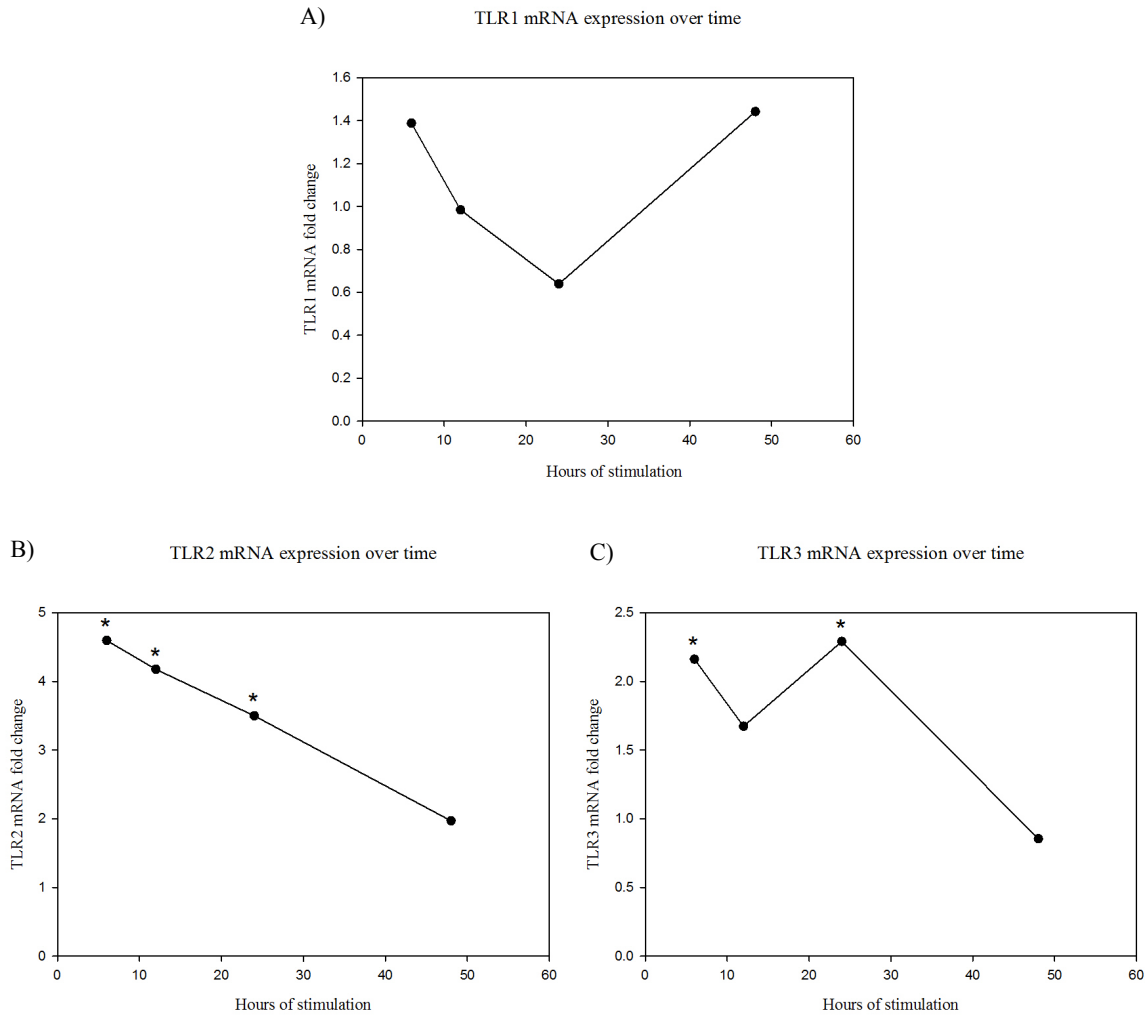

Figure 3.12.1 Time curves of relative gene expressions obtained from REST 2009 using qPCR data from one representative experiment with SW982 cells. The asterisk indicates significant difference from control ($p < 0.05$). Statistical significance was calculated from the total number of biological replicas ($n = 3$). A) The TLR1 mRNA fold changes after 6, 12, 24 and 48 hours of TNF-α stimulation. B) The TLR2 mRNA fold changes after 6, 12, 24 and 48 hours of TNF-α stimulation. C) The TLR3 mRNA fold changes after 6, 12, 24 and 48 hours of TNF-α stimulation.

To summarize, TNF-α treatment resulted in a significant up-regulation of the TLR2 and TLR3 genes. TLR1, TLR4, TLR5, TLR6 and TLR7 expression was not affected. However, TLR1, TLR6 and TLR7 primers showed signs of being nonspecific. Furthermore, the combination treatment with a $cPLA_2$ inhibitor plus TNF-α showed no response in expression levels for any of the TLR genes.

3.13 IL-6 gene expression following TNF-α stimulation

TNF-α is known to increase the IL-6 production in human SFs [85]. Therefore the IL-6 mRNA expression was used to check whether the TNF-α stimulation in the above-mentioned (section 3.11 and 3.12) time-lapse experiments of the SW982 cells had been successful. TNF-α seemed to have increased the IL-6 expression in these experiments (figure 3.13.1), but the 9-fold change was non-significant. Mean fold change and its SEM from the three biological replicas, which the non-significance in this representative experiment is based on, are shown in appendix J.

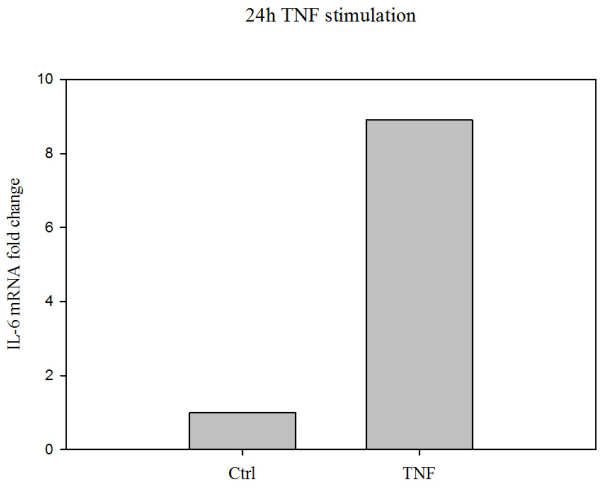

Figure 3.13.1 Relative IL-6 gene expression obtained from REST 2009 using qPCR data from one representative experiment with 24 hours TNF-α stimulation of SW982 cells.

This IL-6 response is considerably weaker than what members of the PLA_2 research group usually detect in TNF-α stimulated SW982 cells. TNF-induced IL-6 fold change values are normally above 20 [86]. The poor IL-6 response in the TNF-α experiments indicates that the stimulation has been non-optimal, and the results may not reflect the reality.

4. DISCUSSION

This master's thesis involves the study of two different pathways believed to be a part of the RA pathogenesis. On the one side, TLR signaling was investigated. On the other side, the effect of the pro-inflammatory cytokine TNF-α, which is a down-stream consequence of TLR activation, was studied. Both regarding TLRs and TNF-α, the aim was to reveal whether there was a connection between these and some of the molecules essential for bone remodeling, and whether the connection involved the $cPLA_2$ enzyme. In addition, the effect of TNF-α stimulation on TLR expression was examined.

All experiments performed during this master's project were done on post-confluent SW982 synovial cells. Post-confluent cells have stopped proliferating due to contact inhibition. Results from flow cytometry, performed by other members of the PLA_2 research group, have shown that 85-90% of the cells are at this point in G_1/G_0 phase of the cell cycle. However, this analysis makes no distinction between the two phases. Assuming that the cells have entered the G_0 phase, they would be in a state of quiescence. As opposed to senescence, apoptosis, and terminal differentiation, quiescence is reversible. The traditional view is that quiescent cells are dormant and have a reduced metabolism. However, recent evidence shows that quiescent cells are in fact exhibiting a special quiescence program, which e.g. involves the expression of genes that enforce this dormant state and genes that ensure the reversibility of the state [87]. Specifically regarding inflammation, it was found that quiescent fibroblasts activate inflammatory responses to a greater extent than proliferative fibroblasts do. Some of the results in this study were that quiescent fibroblasts have a distinctly higher expression of pro-inflammatory genes, such as COX-2, cytokines, CAMs and MMPs, than that of the proliferative ones [88].

4.1 RANKL, OPG and DKK1 gene expression

The RANKL, OPG and DKK1 genes all code for key proteins involved in bone remodeling. Osteoclasts are the cell type responsible for degradation of bone, and RANKL is a component that is necessary for differentiation and function of these cells. OPG is a decoy receptor for RANKL, meaning it can competitively bind RANKL and prevent it from activating osteoclasts. OPG can thus exert protection against bone loss [19].

Osteoblasts are the counterpart to osteoclasts, and they are responsible for the formation of new bone tissue. Osteoblast differentiation and function are dependent on the WNT pathway, and the DKK1 protein functions as an inhibitor of this pathway [19]. In addition, it facilitates bone degradation by decreasing the expression of OPG [26].

Normally there is a balance between bone formation and degradation. In diseases like RA, however, this balance is no longer stable and bone loss is observed. Excessive bone degradation occurs, because the osteoblast function is reduced and the osteoclasts are overly activated. The reason for this seems to be an excess of RANKL relative to OPG. More specifically, it is likely that the RANKL/OPG ratio at the pannus-bone interface, the point where bone erosion occurs, is the determining factor [24]. This is believed to be true since RANKL has been found to be highly up-regulated at pannus-bone interface in RA tissue, while the OPG expression was

minimal here. However, in other synovial membrane cells, away from this interface, the OPG expression was higher [25].

In SW982 cells, OPG mRNA was detected. In addition, RANKL expression may be present, but the qPCR primer for this gene did not show the expected specificity. The C_q values indicate that the RANKL expression is considerably lower than the OPG expression in these cells. Ahmed Siddik was not able to detect RANKL in non-stimulated SW982 cells with the primer used at the time. The PLA_2 research group have used yet another RANKL primer without obtaining signals to any considerable degree in these cells. However, Siv Kristine Sola Strand, another former master's student, succeeded in detecting RANKL in Saos-2 osteoblasts by using the same primer as Siddik, confirming that it was functional. When taking all these RANKL results into account, it would be safe to assume that RANKL is relatively weakly expressed in the SW982 cells. Because the use of the RANKL primer was discontinued, it was not possible to investigate the RANKL/OPG ratio.

The SW982 cell line was established from a synovial sarcoma. and the cells are believed to originate from SFs. SFs from RA patients have been shown to express both RANKL and OPG, with OPG being present in the highest amount [89]. The fact that more OPG was present may indicate that these RA cells were not taken from the pannus-bone interface. The SW982 cells have similarities to these RA SFs, because they too express more OPG than RANKL. This is a further indication that the SW982 cells may originate from SFs, and that this is a suitable cell model for studying RA-related events in the synovium. However, it must be kept in mind that these are cancer cells and can for that exact reason exhibit different gene expressions then their normal state cells.

Furthermore, DKK1 has in recent years been suggested to be just as important in the RA pathogenesis as the RANKL/OPG system. DKK1 is in fact found highly expressed in inflamed synovium, and especially in the SFs [26]. In the SW982 cells, the DKK1 gene is relatively highly expressed – at similar level as OPG. If the translation corresponds with this and the proteins are secreted, it can be assumed that although OPG is produced by RA SFs, similar levels of DKK1 can potentially reduce OPG's bone-protective effect. This will be further discussed in section 4.4.

4.2 TLR gene expression

TLRs are a type of PRRs found on innate immune cells. They recognize various microbial components. In addition, TLRs can bind certain endogenous molecules, such as those found in arthritic joints. Upon activation, the innate immune cells pass on the signal and activate the adaptive immune system. It has been proposed that the TLRs are one of the major contributors in maintaining the inflammatory response in RA [9]. In e.g. RA SFs, TLR2, TLR3 and TLR4 activation have been found to increase the expression of RANKL mRNA and protein, which subsequently promotes osteoclast differentiation [15, 33].

SFs, which are among the key effector cells in RA, are known to express TLR1-6 [5]. Additionally, certain reports have shown elevated levels of TLR7 in RA SFs [8]. There are no published data on the TLR expression in the synovial SW982 cells. Consequently, the TLR1-7 gene expression in these cells was investigated. They were

found to express all the TLRs in question. The average C_q values suggest that TLR2 is the highest expressed TLR in the SW982 cells, closely followed by TLR3. TLR4 and TLR6 are intermediately expressed, while TLR1, TLR5 and TLR7 are moderately expressed. This is somewhat different from the findings in a study done on RA SFs [34]. The highest TLR expression in the RA SFs was found for TLR3, followed by TLR4. TLR2, TLR1 and TLR6 were readily detected as well. TLR5 was barely detectable, and TLR7 was not found in the cells used in this study. Even though the order of the TLR expression levels is not exactly the same in SW982 cells and RA SFs, the similarity is that TLR2, TLR3 and TLR4 are among the most highly expressed TLRs. This also coincides with another finding, which states that RA SFs have especially high levels of functional TLR2, TLR3 and TLR4 [5]. The fact that TLR7 was not detected in the mentioned RA SF study, but has been detected in other studies on RA SFs, shows that even though the same cell type are being analyzed, it does not necessarily give the same results. The reason may be that the RA SFs used were from different locations in the joint, or it may reflect the heterogeneity of the disease. All things considered, the SW982 cells seem like a good model for investigating TLR signaling.

4.3 Suggested TLR signaling pathways in SW982 cells

Before entering a detailed discussion related to TLR agonist stimulation of synovial SW982 cells, an overview of TLR signaling pathways in these cells is presented (figure 4.3.1). In the figure, the red block arrows in front of a component signify what was actually found in the TLR agonist experiments. The signaling proteins and the transcription factor were not investigated in this project, and are consequently based on suggestions or established knowledge. Mapping the signaling proteins involved in these processes in the SW982 cells are issues that can be addressed in further research.

The transcription factor involved in the expression of OPG and DKK1 following TLR1/2, TLR2/6 and TLR3 activation is most likely to be NF-κB. As demonstrated in figure 4.3.1, TLR1/2 and TLR2/6 utilize the MyD88-dependent pathway that results in NF-κB activation [9]. NF-κB activation can also be obtained by TLR3 signaling through the kinase receptor-interacting protein (RIP1) [90]. OPG expression has been found to be dependent of NF-κB in dendritic cells [91], and the DKK1 gene has two NF-κB binding sites in its promoter [92]. Furthermore, the IL-6 and the COX-2 genes, used as positive controls, are known to be NF-κB dependent in SFs [93].

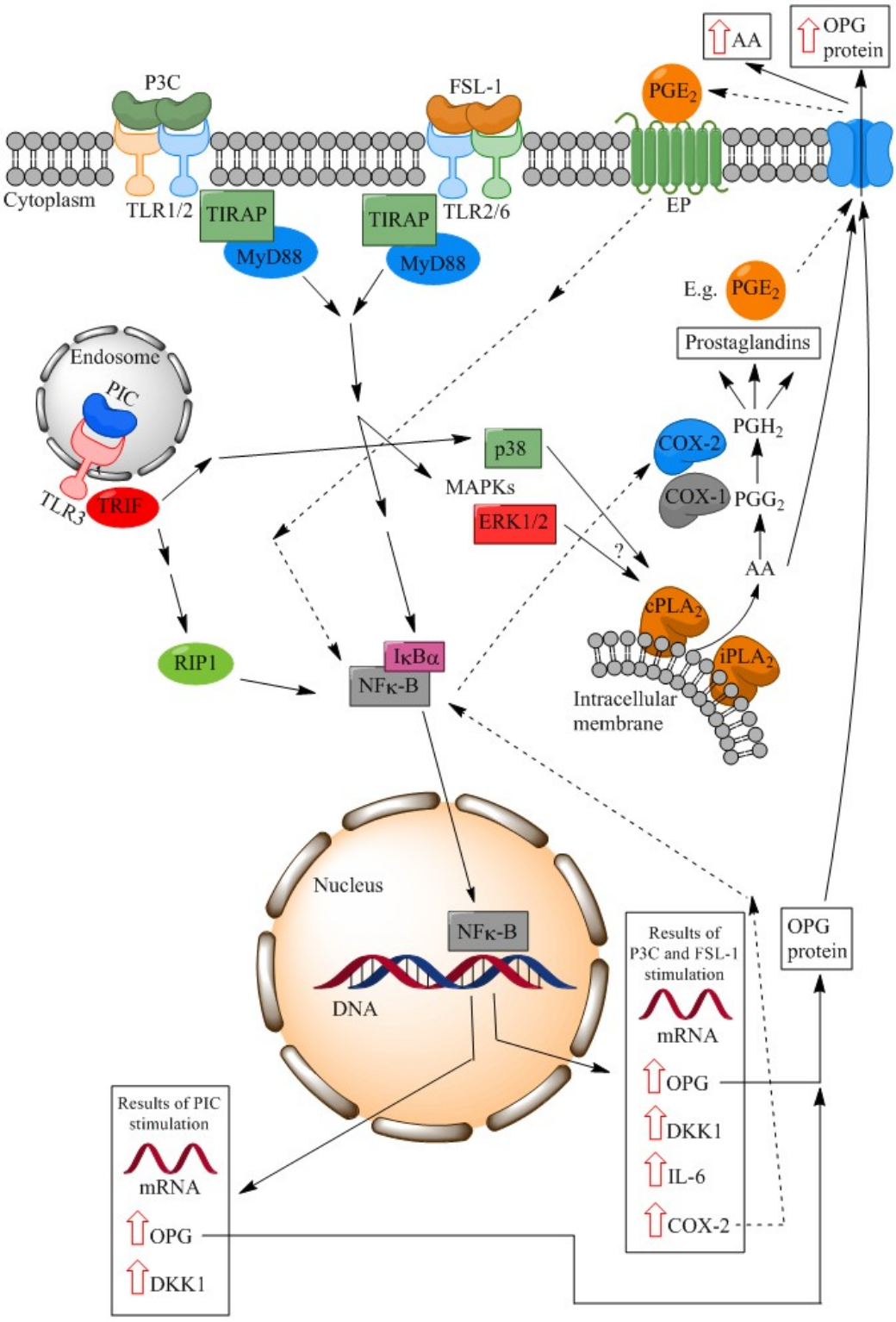

Figure 4.3.1 TLR signaling pathways in SW982 cells activated by the TLR agonists FSL-1, P3C and PIC. The red block arrows in front of a component signify what was actually found in the TLR agonist experiments, and they symbolize an increase of the respective component. The dashed arrows are only suggested connections, while the solid ones symbolize connections found in the literature. AA, arachidonic acid; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DKK1, dickkopf homolog 1; EP, prostaglandin E_2 receptor; ERK, extracellular-signal regulated kinase; FSL-1, synthetic diacylated lipoprotein; IκBα, inhibitor of NF-κB; IL, interleukin; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; IRF, interferon regulatory factor; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary-response protein-88; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; P3C, the synthetic triacylated lipoprotein Pam3CSK4; PG, prostaglandin; PIC, the synthetic dsRNA analog Poly(I:C); RIP1, receptor interacting protein 1; TIRAP, TIR domain-containing adaptor protein; TRIF, TIR domain-containing adaptor-inducing IFN-β

4.4 OPG and DKK1 gene expression in response to TLR agonists

As mentioned in section 4.2, stimulation of TLR2, TLR3 and TLR4 have been shown to increase the RANKL expression in RA SFs. Increased OPG expression has been detected after stimulation of TLR3 and TLR4 [91]. However, this was in dendritic cells. No published data were found regarding a connection between TLR activation and DKK1. Having found that the SW982 cells express all TLRs from 1 to 7, the next step was to explore whether there was a connection between TLR activation and the expression of RANKL, OPG and DKK1. As previously stated, the RANKL qPCR primer did not function as expected, hence only OPG and DKK1 were investigated.

Upon stimulation with the TLR2/6 agonist FSL-1, the TLR1/2 agonist P3C, and the TLR3 agonist PIC, it was found that the SW982 cells increase their OPG gene expression. This increase was significant for all TLR agonists after a stimulation period of 9 hours. These findings coincide with the dendritic cell study, where stimulation of certain TLRs resulted in elevated OPG levels. Regarding the disrupted RA bone homeostasis, an OPG increase would be a positive event, as OPG binds RANKL and thereby reduces activation of bone-degrading osteoclasts. Because the TLRs are a part of the innate defense mechanisms, it is not surprising that they will activate protective responses, such as increased OPG. However, upon continuous activation of TLRs due to endogenous ligands, which is believed to occur in RA, there would probably be too much OPG produced. There is a possibility that an excess of OPG could lead to additional, unwanted effects, such as increased proliferation or reduced apoptosis of RA effector cells. In addition to being a decoy receptor for RANKL, OPG has been found to function as an antagonizing receptor for TNF-related apoptosis-inducing ligand (TRAIL), hence preventing apoptosis [94]. Furthermore, it has been demonstrated that this is happening in cultured RA SFs [95]. Elevated levels of OPG due to IL-1β stimulation caused reduced apoptosis of RA SFs, indicating a role for OPG in the synovial hyperplasia observed in RA. This may be something to investigate further in the SW982 cells, by e.g. treating the cells with external OPG and observe their growth.

In initial experiments with 6 hours TLR agonist stimulation, P3C resulted in a smaller OPG induction than FSL-1, and PIC even less. FSL-1 generally showed a stronger induction than P3C for several of the responses studied. This may be due to the higher expression of TLR6, than TLR1, in the SW982 cells. If this is also shown to be true at protein level, it can be assumed that the more receptor present, the stronger the agonist response. However, after several biological replicas of 9 hours stimulation, the OPG fold induction mediated by FSL-1 and P3C was leveled. That the initial experiments showed minimal PIC induction, do not coincide with the finding that TLR3 expression seems to be fairly high in the SW982 cells. TLR3 is an intracellular receptor, and it may be assumed that PIC would need a longer stimulation period to evoke the same amount of response as FSL-1 and P3C. However, 24 hours PIC stimulation resulted in even less OPG induction than after 9 hours. Moreover, PIC negatively affected the SW982 cells' morphology. The cells did not seem to thrive, and apoptosis was suspected. A lot more cells than usual loosened from the cell culture plate, and the ones remaining adherent adopted an abnormal morphology. The cells are usually thinner and longer upon serum starvation and stimulation, as this stresses the cells. With PIC stimulation, the cells were even thinner and there was more space between them. As the PIC stimulation period increased, more cells loosened. As a consequence, the PIC concentration was reduced. The lower

concentration (5 μg/ml) was better tolerated by the cells, but they still adopted a thinner shape and loosened from the culture plate. The SW982 cells are obviously very sensitive to this viral dsRNA analog. The PIC-mediated OPG response, and the other responses detected following PIC stimulation, may be due to apoptotic stress. However, according to the aforementioned dendritic cell study, an OPG up-regulation is anticipated upon TLR3 activation.

The pro-inflammatory cytokine, IL-1β, used as positive control stimulation, induced OPG expression in the SW982 cells, in comparable levels to that of FSL-1. IL-1β is known to contribute to inter alia bone erosion in RA, but the cytokine has been shown to increase both OPG mRNA and protein levels in RA SFs [95, 96]. This illustrates the complexity of cytokine effects. Even though IL-1β increases the expression of the bone-protecting OPG in RA, this is not adequate to counteract other processes favoring bone loss. However, the OPG up-regulation following IL-1β stimulation of RA SFs can also function to prevent apoptosis, as previously discussed.

Furthermore, FSL-1, P3C and PIC were found to induce DKK1 gene expression in the SW982 cells, although the response was generally weaker than for OPG. Because the DKK1 gene promoter has NF-κB binding sites [92] and the TLRs in question are known to activate this transcription factor, an increase in DKK1 expression is expected. Elevated DKK1 levels will lead to less activation of osteoblasts and hence reduced bone formation, consequently tipping the bone homeostasis towards degradation. More specifically, since there are fewer active osteoblasts, there will be less OPG production. This will consequently lead to more RANKL available and increased osteoclast activity. In addition, DKK1 expression in osteoarthritic SFs has been found to increase the production of angiogenic factors and cartilage degrading components [97], which both are hallmarks of arthritis. Thus, DKK1 seems to have several negative effects in RA.

As for OPG, 9 hours was the stimulation period resulting in significant increase in DKK1 gene expression for all TLR agonist. After 9 hours stimulation, the DKK1 increase was similar for FSL-1 and P3C, while PIC resulted in a higher induction. PIC showing the highest induction is the opposite of that seen for OPG. This suggests that the intracellular TLR3 activation, and subsequent activation of NF-κB, has a greater effect on the DKK1 expression. Again, this may be a result of apoptotic stress mediated by PIC.

The positive control, IL-1β, increased the DKK1 expression of the SW982 cells, and this is also found to be the case in arthritic SFs. However, it has been demonstrated that IL-1β do not induce DKK1 directly. It increases the expression of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) that activates glucocorticoids, which then again induce DKK1 expression [98].

In summary, all TLR agonists in question cause elevated mRNA levels of both OPG and DKK1 in SW982 cells. In a RA joint, it can be imagined that even though expression of OPG increases and reduce bone degradation, similar levels of DKK1 may counteract this by preventing bone formation and OPG expression by osteoblasts. Specifically in the SFs, OPG may exert negative effects in a RA context by preventing apoptosis of these cells, hence contributing to synovial hyperplasia.

Moreover, DKK1 makes the SFs express angiogenic and cartilage degrading factors, which both are involved in RA.

4.5 TLR activation and secretion of OPG protein

The OPG protein analyses of cell culture supernatants, collected after 9 hours TLR agonist treatment, revealed the same trends as seen at mRNA level in the initial 6 hours experiments. All TLR agonists resulted in OPG protein induction. The FSL-1- and IL-1β-mediated increase in OPG protein level was similar, and significantly higher than for both P3C and PIC. P3C seemed to evoke a slightly stronger induction than PIC, but the difference was not significant. The mRNA trend following 6 hours of stimulation coincided with the protein trend after 9 hours stimulation. This is anticipated, as the protein response always will be somewhat delayed relative to the gene response. The same trend was found in the first gene analyses after 9 hours stimulation. However, the more biological replicas performed, the more similar FSL-1 and P3C response was observed. There was a hint of this tendency in an initial experiment where supernatants were collected after 12 hours stimulation. However, several replicas and inhibitor treatments were only performed with 9 hours stimulation periods, and therefore the supernatants were collected at this stimulation time.

Taken together, OPG protein secretion increases due to TLR activation in the SW982 cells. This may also be the case in SFs under RA development, as these cells are believed to originate from SFs and TLRs are known to be activated in RA. OPG protein has in fact been detected in culture supernatants of RA SFs, and it was increased upon IL-1β stimulation [95]. A local increase of OPG protein in the joint will bind more RANKL, consequently causing less osteoclast activation and thereby less bone degradation. This may contribute to restoration of bone homeostasis. As previously mentioned, OPG have been shown to antagonize TRAIL-induced apoptosis in RA SFs, and thereby being partly responsible for the expansion of RA synovial lining [95]. OPG up-regulation is not only a positive thing, and the extent of OPG's bone protective effect depends on the RANKL amount present in the joint, and probably also the DKK1 amount. Hence, the events are very complex and cannot be evaluated separately. Therefore, RANKL and DKK1 protein levels should be investigated, and all cells' contribution should be considered.

4.6 OPG gene expression and the prostaglandin pathway

PGs are lipid mediators in the eicosanoid family, and they have a key role in inflammation [40]. Studies have demonstrated that PGE_2 down-regulates OPG mRNA levels in human bone marrow stroma cells [99], and in mouse osteoblasts after LPS and IL-1 stimulation [50]. These findings indicate that the $cPLA_2$ enzyme, which is responsible for producing the first precursor for PGE_2, is involved in regulating the OPG expression. This implies that inhibiting the $cPLA_2$ and COX enzymes may lead to less PGE_2 and consequently more OPG may be expressed. In the 9 hours TLR agonist stimulation experiments performed in this master's project, FSL-1-mediated OPG gene expression did not seem to change upon inhibition of the PG pathway in the SW982 cells. The $cPLA_2$ inhibitors and the COX inhibitor resulted in a more distinct trend of inhibitory effect on the P3C-mediated OPG expression. However, only the COX inhibitor had a significant effect. This suggests that activation of TLR1/2 by P3C leads to increased activity of the COX enzymes – whether it is COX-

1, COX-2, or both is impossible to tell due to the fact that Indomethacin is a nonspecific COX inhibitor. However, it is most likely to be COX-2, as COX-2 is the inducible isoform [83]. COX-2 expression was in fact found to be highly elevated by TLR agonist treatment of SW982 cells. Consequently, elevated levels of COX-2 would cause an increase in PG levels, which might be one of the factors leading to the observed P3C-induced OPG mRNA increase. This is, however, the opposite of what was found in the above-mentioned bone marrow stroma cells and mouse osteoblasts studies [50, 99], where increased PGE_2 levels resulted in a down-regulation of OPG. The difference may be attributed to variations between cell types, and the fact that PGE_2 has several different receptors and can therefore evoke different responses [48].

In the OPG protein analyses of SW982 supernatants following 9 hours stimulation, minimal effects of the $cPLA_2$ inhibitors and the COX inhibitor were detected – none were significant. Thus, the significant reduction of P3C-mediated OPG gene expression by COX was not found at protein level. This change could possibly have been detected at protein level after 12 hours stimulation. However, 12 hours inhibitor experiments were not performed, because 9 hours was the optimal stimulation period for TLR agonist induction of OPG and DKK1. Hence, the current results are not adequate to confirm that the PG pathway is involved in TLR agonist-induced OPG expression.

4.7 Prostaglandin pathway involvement in DKK1 gene expression

There is evidence suggesting that the WNT pathway in osteocytes is triggered by crosstalk with the PG pathway [100]. That is, PGE_2 can activate the WNT pathway, which causes a decrease in the expression of WNT inhibitors like DKK1. However, there are no published data describing a connection like this in SFs.

In the case of SW982 cell stimulation with FSL-1, it is unclear whether the PG pathway is involved, as the DKK1 increase was non-significant and only the COX inhibitor resulted in significant DKK1 inhibition. For P3C, however, both $cPLA_2$ inhibitors and the COX inhibitor reduced the DKK1 gene expression to levels similar to that of untreated cells. This suggests that the PG pathway is indeed involved in the increased DKK1 expression observed when the cells are treated with P3C. However, this is the opposite of what was found in osteocytes, where PGE_2 release resulted in decreased DKK1 expression.

The results discussed this far indicate that TLR1/2 signaling causes both increased OPG and DKK1 gene expression, and that this probably is due to activation of the PG pathway – at least in case of DKK1. The fact that the PG pathway contributes to the DKK1 increase, indicates that $cPLA_2$ inhibitors may be a promising RA treatment by reducing the expression of this negative effector. If the PG pathway is in fact involved in TLR1/2-mediated OPG expression too, $cPLA_2$ inhibition would be detrimental in respect to reducing OPG's bone protection, but favorable in respect to reducing OPG's apoptosis hindrance in RA SFs. All effects of $cPLA_2$ inhibition, both positive and negative, need to be determined and assessed in a holistic manner. Therefore, $cPLA_2$'s role in RA events must be further elucidated.

4.8 IL-6 and COX-2 gene expression following TLR activation

Gene expression analysis of IL-6 and COX-2, which are highly inducible genes in the SW982 cells, were performed to ensure that the TLR agonist stimulation had been successful. The findings can also be used for clarifying the TLR signaling events in these cells. Both IL-6 and COX-2 are involved in RA pathogenesis. The pro-inflammatory cytokine IL-6 is prominent in RA synovial fluid [101], and COX-2 enzyme is found to be elevated in synovial tissue from RA patients [102].

FSL-1 and P3C have previously been found to induce IL-6 expression in human gingival fibroblasts [103]. Specifically in RA SFs, it has been demonstrated that ligands for TLR2-5 evoke IL-6 production [34]. Furthermore, IL-1β-induced IL-6 production by RA SFs has been shown to be significantly reduced by the COX-2 inhibitor NS-398 [83], indicating that the PG pathway is involved. In the SW982 cells, both FSL-1 and P3C resulted in abundant IL-6 induction. These stimuli resulted in fold changes around 3 for OPG and DKK1, while the IL-6 fold change value was above 30. Hence, it can be concluded that the TLR agonist stimulation was successful. Regarding the inhibitors, only the COX inhibitor resulted in significant reduction of FSL-1-mediated IL-6 expression. For P3C-mediated IL-6 expression, on the other hand, both $cPLA_2$ inhibitors had significant reducing effects. These results suggest that the PG pathway is involved in the IL-6 production following TLR1/2 activation, and possibly following TLR2/6 activation. A potential explanation to why the $cPLA_2$ inhibitors failed to reduce FSL-1-mediated IL-6 expression, is that the response actually is too strong and that an increased inhibitor concentration is required. The FSL-1-mediated IL-6 fold change was markedly higher than the P3C-mediated.

TLR2/6 and TLR1/2 ligands have been shown to induce COX-2 levels and activity in osteoblasts from mice [104]. This was confirmed by a distinct increase in PGE_2 levels in the culture medium. Furthermore, the TLR2/6 and TLR1/2 ligand treatments showed an activation of the transcription factor NF-κB in the osteoblasts. In SW982 cells, TLR agonist and inhibitor treatment resulted in the same COX-2 up-regulation trend as for IL-6: FSL-1 showed the strongest COX-2 induction, and only the COX inhibitor reduced this significantly. COX-2 expression increased after P3C stimulation, and both $cPLA_2$ inhibitors plus the COX inhibitor had significant reducing effects. These results indicate that the FSL-1- and P3C-mediated COX-2 expression exerts a positive feedback-loop, because inhibition of the PG pathway leads to reduced COX-2 mRNA. I.e., more production of PGs by the COX-2 will in absence of the COX inhibitor promote further COX-2 expression. In conclusion, a connection obviously exists between TLR activation and the PG pathway in the SW982 cells, which is equivalent to the findings in mouse osteoblasts.

4.9 AA release in response to TLR agonists

AA is the first precursor for eicosanoids, such as PGs. AA is incorporated in cell membranes and is released by $cPLA_2$, $sPLA_2$, $iPLA_2$ or $LpPLA_2$ [52]. Which PLA_2s that participate in catalysis of AA release varies between cell types. GIVA $cPLA_2$ is specific for AA release at the *sn-2* position and is considered to be the major AA releasing enzyme [40]. The GIVA cPLA2 is activated by MAPKs, such as ERK1/2 and p38. TLR3 and TLR4 signaling has been reported to activate both these MAPKs in RA SFs [105]. There are no published data on other TLRs' connection to AA

release in human SFs. However, it has been demonstrated that stimulation of TLR1/2, TLR2, TLR3, TLR4, TLR2/6, and TLR7 in mouse macrophage-like cells leads to elevated levels of AA release [106].

Here, activation of TLR2/6, TLR1/2, and TLR3 all resulted in a significant increase in AA release from the SW982 cells. The positive control, IL-1β, increased the AA release as expected. The AA responses in the SW982 cells were time-dependent for all TLR agonists. AA release following FSL-1, P3C and IL-1β stimulation peaked after 9 hours, while PIC-mediated AA release peaked after 12 hours. This may reflect the fact that PIC needs more time for reaching its maximum induction, even though this is not beneficial to the cells judged by their abnormal morphology, as previously discussed. P3C- and PIC-induced AA release were similar, while FSL-1 was the agonist resulting in the highest response. The FSL-1 AA fold change was similar to that of IL-1β. The trend that FSL-1 is the strongest inducer is the same as observed at gene level for IL-6 and COX-2, and at protein level for OPG. The SW982 cells' responses are a little different from that of the mouse macrophage-like cells. In the mouse cell study, FSL-1 and P3C resulted in similar and intermediate AA release, while PIC stimulation showed the strongest response. The different responses may simply be due the fact that they are different cell types, from different species, and therefore react differently. That being said, the mouse cell study used higher concentrations for all TLR agonists: 1 μg/ml for FSL-1 and P3C (vs. 100 or 50 ng/ml for FSL-1 and 100 or 300 ng/ml for P3C), and 25 μg/ml for PIC (vs. 5 or 1 μg/ml). In addition, a different stimulation period was used: 20 hours (vs. 3, 6, 9, 12 and 24 hours). Regardless, elevated levels of AA release may cause increased AA uptake by neighboring cells. This will provide more substrates for the COX enzymes, which ultimately leads to increased PG production and thereby stronger inflammatory reactions.

Furthermore, experiments using different PLA_2 inhibitors were performed in order to reveal which enzymes were responsible for the observed TLR agonist-induced AA release in the SW982 cells. FSL-1, P3C and PIC all resulted in similar AA release trends when combined with the inhibitors. The GIVA $cPLA_2$ inhibitor AVX002 had significant inhibitory effects for both FSL-1 and P3C. This $cPLA_2$ inhibition was confirmed by the use of CAY10502 that seemed to be even more effective and reduced the AA release to basal level. Even though AVX002 showed a tendency of inhibition for PIC as well, this was found to be non-significant. However, the other GIVA $cPLA_2$ inhibitor, CAY10502, resulted in significant reduction to basal level for PIC. These results strongly indicate that the GIVA $cPLA_2$ is the main PLA_2 contributing to the TLR agonist-induced AA release. For all three TLR agonists, neither of the $sPLA_2$ inhibitors, CAY10590 and Varespladib, had significant inhibitory effect on the AA release. This suggests that $sPLA_2$ is not involved in AA release in response to TLR agonists. MAFP, which inhibits both $iPLA_2$ and $cPLA_2$, reduced the AA release to basal level for all TLR agonists. This could be due to the $cPLA_2$ inhibition alone, or due to both $cPLA_2$ and $iPLA_2$ inhibition. From the results using the $iPLA_2$ inhibitor, BEL, the involvement of $iPLA_2$ can be evaluated. BEL showed significant inhibition for FSL-1 and P3C, but non-significant for PIC. It can be assumed that $iPLA_2$ is partly involved in the AA release induced by TLR agonists. However, BEL has also been found to inhibit cellular phosphatidic acid phosphohydrolase (PAP) -1 [107]. PAP-1 catalyzes the conversion of phosphatidic acid on membrane surfaces to inorganic phosphate and diacylglycerol (DAG). DAG

can then activate protein kinase C (PKC), which in turn have been found to contribute to GIVA $cPLA_2$ activation upon e.g. TLR4 agonist stimulation [108]. Thus, the decreased AA release following BEL treatment of SW982 cells can possibly be attributed to PAP-1 inhibition. This should be further elucidated to find out whether BEL is actually acting on $iPLA_2$ under the conditions used here (10 μM inhibitor).

In the aforementioned study [106], where TLR stimulation led to increased AA release in mouse macrophage-like cells, it was found that GIVA $cPLA_2$ is the key enzyme regulating AA release. It was suggested that GV $sPLA_2$ contributed to the TLR-mediated release by amplifying the activation of GIVA $cPLA_2$ via ERK1/2 phosphorylation. As in the mouse macrophage-like cells, the TLR-mediated AA release is mainly due to the action of GIVA $cPLA_2$ in the SW982 cells. In addition, the SW982 cell results indicate a possible involvement of $iPLA_2$. The mouse cell study found no role for $iPLA_2$, but for $sPLA_2$. The $sPLA_2$ should not be ruled out just yet in the SW982 cells. A possible reason for the lack of $sPLA_2$ inhibitor effects can be the long pre-incubation period. 2 hours pre-incubation was used for all inhibitors. This may be too long for the $sPLA_2$ inhibitors to keep their proper function. In contrast, the mouse cell study only used a 30 minutes pre-incubation period, although they used another $sPLA_2$ inhibitor (scalaradial) and $cPLA_2$ inhibitor (pyrrophenone). Furthermore, the study stated that they had performed an extensive characterization of the inhibitors' effect on the cells, and ensured that the inhibitor concentrations used did not interfere with other PLA_2 activities than those the inhibitors are directed against. A similar analysis would be required for the SW982 cells, to find out whether the observed effects are in fact due to the PLA_2s suggested here.

SW982 cells' OA release was investigated in parallel to AA release, since certain cell models show an increase in OA release too, e.g. in response to the TLR4 ligand LPS [42]. OA release reflects the activity of AA-non-selective PLA_2s. The TLR agonist stimulation generally resulted in unchanged OA release in the SW982 cells. If there was a change, this was negligible compared to the AA fold change. The same goes for the experiments with the different PLA_2 inhibitors. Although this could suggest that the TLR agonists do not activate non-selective PLA_2s, the BEL effect on TLR-mediated AA release indicates that $iPLA_2$ is activated. The reason for not detecting this effect in the OA release is probably that this AA/OA assay is not sensitive enough to detect changes in OA levels.

4.10 TNF-α effects on RANKL, OPG and DKK1 gene expression

As a consequence of TLR activation, several pro-inflammatory mediators are produced, including cytokines. TNF-α is a cytokine considered especially important in RA pathogenesis, as it leads to e.g. angiogenesis and activation of leukocytes, endothelial cells, and SFs [4]. Regarding bone remodeling components, TNF-α has been found to induce RANKL mRNA expression in SFs from RA patients [109]. However, the same study also showed that these RA SFs expressed OPG and that the OPG mRNA expression was further enhanced by TNF-α stimulation, suggesting a bone-protective role for TNF-α. This has been demonstrated in other studies as well [20, 110]. These findings are probably related to the fact that TNF-α exhibits different roles during different disease stages: pro-inflammatory in the initiation phase and disease-suppressive at a later stage. In addition, the OPG/RANKL ratio is the critical determinant for whether TNF-α causes bone degradation or protection against this

[109]. It must be stated again that OPG increase is not necessarily beneficial in a RA context. It is indeed positive in respect to bone homeostasis, but it can also prevent TRAIL-induced apoptosis of RA SFs [95]. I.e., OPG is considered a partaker in synovial hyperplasia.

Furthermore, DKK1 has been found to be elevated in response to TNF-α in arthritic SFs [24]. More recent findings showed that, as for IL-1β, it is not TNF-α directly that causes the elevated DKK1 levels in RA SFs. It is, however, its contribution in increasing the expression of the enzyme 11β-HSD1, which activates glucocorticoids. It is the glucocorticoids that actually induce the DKK1 expression [98]. Whether $cPLA_2$ is involved in expressing 11β-HSD1 may also be an area to explore in the SW982 cells.

TNF-α time-lapse experiments performed on the SW982 cells were not conclusive. No significant OPG or RANKL induction was observed. Ahmed Siddik, the former master's student, was able to detect a small, but significant increase in OPG gene expression in response to TNF-α, but was not able to detect RANKL in non-stimulated SW982 cells. The RANKL primers used then did not work in qPCR, but conventional PCR indicated the presence of RANKL upon TNF-α stimulation. Therefore, it was expected to observe RANKL and OPG fold changes with the new primers. However, three biological replicas showed no significant changes. The trends in the different replicas differed, and it was not possible to determine potential outliers. The results may be influenced by differences in generation numbers on the cell cultures used for experiments. Cells used in two of the replicas were close in generation number (#67 and #69), but one replica was different (#37) and from another cell batch. Cell lines do not show signs of senescence. However, they may change their gene expression profile as the number of cell division cycles increases. No obvious trend for this was recognized in the TNF-α experiments. Regardless, the experiments with the TLR agonist stimulations were performed on cell cultures close in generation number.

Another indication of non-optimal TNF-α experiments was the IL-6 gene analysis that revealed a rather small induction. The fold change was around 9, and also non-significant. Moreover, this is a considerably smaller induction than what the PLA_2 research group normally obtains in SW982 cells. The IL-6 fold change values are usually above 20 in response to TNF-α [86]. The weak response might have been because of an impaired batch of TNF-α, or because the cells were abnormal due to e.g. contamination. In addition, it was in these TNF-α experiments the use of RANKL, TLR1, TLR6 and TLR7 primers was discontinued due to indications of non-specific products.

Despite the varying results, a significant increase in DKK1 gene expression was detected after 6 hours of TNF-α stimulation. An increase was anticipated based on mentioned findings in RA SFs, and based on Siddik's findings in SW982 cells. If this increase in DKK1 mRNA corresponds to an increase of DKK1 protein and secretion of this, as it does in RA, this will lead to increased inhibition of the WNT pathway and consequently less osteoblast activation. As indicated several times, whether this causes an unbalance of bone homeostasis also depends on the RANKL/OPG ratio. Thus, this ratio needs to be evaluated, but this was not possible based on the results obtained from these experiments.

A study of human dental pulp cells has showed that a $cPLA_2$ inhibitor reduces the increase of RANKL mRNA levels induced synergistically by TNF-α and IL-1-α [111]. To investigate whether there is a connection between $cPLA_2$ and TNF-α-induced RANKL, OPG and DKK1 in SW982 cells, the TNF-α time-lapse experiments included the $cPLA_2$ inhibitors AVX002 and InhibX. The latter is a novel inhibitor whose structure has not yet been published, and InhibX is a cover name used in this master's thesis. Neither AVX002 nor InhibX showed significant effects on any of the bone remodeling components. Because these TNF-α experiments were inconclusive, it is not possible to conclude whether these no-response $cPLA_2$ inhibitor results are actually valid. Hence, the experiments would need to be repeated to evaluate $cPLA_2$'s involvement in TNF-α-induced expression of RANKL, OPG and DKK1.

A desired scenario is that the $cPLA_2$ enzyme was found to up-regulate the expression of both DKK1 and RANKL in response to TNF-α. Consequently, this would strengthen the $cPLA_2$ inhibitors' candidacy as anti-rheumatic drugs aiming to reestablish the bone homeostasis. Furthermore, if an OPG increase following TNF-α treatment had taken place, it would not have been beneficial to the bone homeostasis if the $cPLA_2$ inhibitors reduced the OPG expression. However, it would have been advantageous in respect to increasing the apoptosis of RA SFs. Again, it is essential to map all negative and positive effects of $cPLA_2$ inhibition.

4.11 TLR gene expression following TNF-α stimulation

As previously stated, the expression of cytokines like TNF-α is a result of TLR signaling. There is evidence that TNF-α affects the TLR expression. E.g., in RA SFs, TNF-α was found to increase TLR2 expression [34]. The same TNF-α time-lapse experiments as discussed in the previous section were analyzed at gene level for TLR expression, to investigate TNF-α's effect on TLR1-TLR7 in SW982 cells. As for OPG and RANKL, the TLR results varied and there were few significant changes. Only TLR2 and TLR3 gene expression were significantly increased. For both these TLRs, short TNF-α stimulation periods, such as 6 and 12 hours, resulted in the highest induction. Moreover, the TLR2 response seemed to be stronger than the TLR3 response. Although none of the other TLRs showed significant changes in gene expression, the possibility of them being affected by TNF-α cannot be excluded solely based on these experiments. As stated in the previous section, the induction of IL-6 was unusually poor, and this indicates that the TNF-α stimulation has not been optimal. I.e., repetition is necessary to verify these results. However, the SW982 results coincide with the previously mentioned RA SFs study [34], regarding TNF-α increasing the TLR2 expression. Moreover, the RA SFs study found that TLR3, TLR4 and TLR5 expression were unaffected by TNF-α. This seems to be the case for SW982 cells too, except that TLR3 was indeed affected.

It could be imagined that TNF-α-induced TLR expression could contribute to a vicious circle in RA. Activation of certain TLRs would lead to more TNF-α, which then again would cause an increase in TLR expression. The cells would then be more vulnerable to endogenous TLR ligands present in the inflamed joint, and subsequent TLR activation would produce a stronger immune response. This would involve increased TNF-α expression and the circle would go on.

Furthermore, TNF-α-induced TLR2 expression has been linked to inter alia $cPLA_2$ activation in human gingival fibroblasts in a study associated with the chronic inflammatory disease periodontitis [112]. In the SW982 cells, the $cPLA_2$ inhibitors, AVX002 and InhibX, had no significant effect on the TNF-α-induced TLR expression. Again, the results are inconclusive and $cPLA_2$'s involvement should not be ruled out just yet.

4.12 Limitations of cell culture experiments

When working with cell cultures in general, it must be kept in mind that there is a risk of deceptive results when cells are removed from their physiological environment. Inside the body, the behavior of the cells is highly dependent on interactions with other cell types and the extracellular matrix. These components are obviously not present in an *in vitro* two-dimensional (2D) culture and are therefore not fully representable for what happens in the body [113]. Moreover, in cell culture experiments, the researcher has limited spatial and temporal control regarding e.g. addition of stimuli. An up-and-coming alternative to 2D cell cultures is the so-called cell chips. These are microsystems that can provide both a more authentic three-dimensional (3D) environment for the cells and more controllable ways of regulating transport of fluids and soluble factors [114]. In addition, microsystems enable single cell studies, which may be a more correct way of analyzing cells' responses to e.g. certain compounds. Cells in a culture are often assumed to be homogeneous, and the average response of the cells is then considered as the response of all cells in that culture. The fact is, that even though cells are of the same type, they can be different from each other. This heterogeneity should be taken into consideration [115] .

4.13 Future research

All the SW982 cell experiments performed in this master's project should be performed on a different SF cell line as well, to exclude the risk of obtaining cell-specific effects and to ensure that the results are physiologically relevant. Optimally, the results should be verified in primary SFs to ensure that the observed effects are not due to the cancer state of the cell line. Even though the TLR agonist stimulation results must be verified, this study of TLR signaling can serve as a valuable guidance for further research concerning the connection between TLRs, $cPLA_2$, the PG pathway, bone remodeling components and pro-inflammatory mediators in RA. Because the SW982 cells express TLR1-TLR7, additional TLR agonists (than only the TLR2/6, TLR1/2 and TLR3 agonists) should be included in further studies. Examining DAMP-mediated TLR signaling to see whether this will evoke different responses than PAMP-mediated, would also be of interest. Furthermore, possible expression of other human TLRs (TLR8-TLR10) should be investigated in the SW982 cells. The experiments regarding the involvement of $cPLA_2$ in expression of RANKL, OPG, DKK1 and TLRs in response to TNF-α were inconclusive, and should be repeated.

Finally, it should be mentioned that the gene expression analysis software REST 2009 (Qiagen) used in this thesis, is no longer subject to further development. In future gene studies it should therefore be considered to use another program, e.g. qbase+ (Biogazelle). This program has more functions integrated and will make it easier to do extensive qPCR data-analysis.

5. CONCLUSION

This thesis involves the study of synovial SW982 cells' responses to TLR agonists and TNF-α, and the involvement of the $cPLA_2$ enzyme in the expression of the bone remodeling components RANKL, OPG and DKK1. It was demonstrated that SW982 cells express OPG and DKK1, and probably RANKL. In addition, the cells were found to express TLR1-TLR6, and most likely TLR7 as well.

Furthermore, stimulating the cells with a TLR2/6 agonist (FSL-1), a TLR1/2 agonist (P3C), or a TLR3 agonist (PIC) resulted in an increase of OPG gene expression and consequently more OPG protein secretion, which could potentially prevent osteoclast activation. However, this would depend on the amount of RANKL present, but this was not possible to determine due to a nonspecific RANKL primer. In addition, the TLR agonists induced higher levels of DKK1 gene expression. If this would correspond to an increase in DKK1 translation, secreted DKK1 would inhibit osteoblast activation and thereby reduce OPG expression. FSL-1 and P3C stimulation highly increased the gene expression of COX-2 and IL-6, which both are pro-inflammatory mediators.

The gene expression trends indicated that the PG pathway (i.e. $cPLA_2$ and COX enzymes) is involved in P3C-induced expression of DKK1, IL-6 and COX-2, but possibly OPG as well. For FSL-1-induced expression of these genes the involvement of the PG pathway is not as evident as for P3C, but still likely. PIC-induced expression was not studied at gene level with $cPLA_2$ and COX inhibitors.

FSL-1, P3C and PIC all increased the release of the inflammatory intermediate AA – the first precursor of PGs. The GIVA $cPLA_2$ enzyme was found to be the main PLA_2 responsible for this AA release, with a possible involvement of $iPLA_2$.

Even though the bone-protective OPG is up-regulated by activation of TLR2/6, TLR1/2 and TLR3, this activation may have more negative effects in a RA context as the levels of DKK1, COX-2, IL-6 and AA release are also increased. In addition, an OPG increase may contribute to synovial lining hyperplasia due to prevention of SF apoptosis. Thus, from the results in this study, $cPLA_2$ seems like an attractive target for inhibiting inflammatory responses set off by TLRs.

The SW982 cells were found to up-regulate their DKK1, TLR2 and TLR3 gene expression in response to the pro-inflammatory cytokine TNF-α, which is one of the components produced following TLR activation. The no-response results for RANKL, OPG, TLR1, and TLR4-7 are inconclusive, partly because the TNF-α stimulation was found to be non-optimal. The same goes for the no-response results regarding $cPLA_2$'s involvement in TNF-α-induced expression of the mentioned genes.

References

1. Smolen, J.S., et al., *New therapies for treatment of rheumatoid arthritis.* The Lancet, 2007. **370**(9602): p. 1861-1874.
2. McInnes, I.B. and Schett, G., *MECHANISMS OF DISEASE The Pathogenesis of Rheumatoid Arthritis.* New England Journal of Medicine, 2011. **365**(23): p. 2205-2219.
3. Smolen, J.S. and Steiner, G., *Therapeutic strategies for rheumatoid arthritis.* Nature Reviews Drug Discovery, 2003. **2**(6): p. 473-488.
4. McInnes, I.B. and Schett, G., *Cytokines in the pathogenesis of rheumatoid arthritis.* Nature Reviews Immunology, 2007. **7**(6): p. 429-442.
5. Bartok, B. and Firestein, G.S., *Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis.* Immunological reviews, 2010. **233**: p. 233-255.
6. Mikuls, T.R., *Co-morbidity in rheumatoid arthritis.* Best Practice & Research Clinical Rheumatology, 2003. **17**(5): p. 729-752.
7. Kindt, T.J., et al., *Immunology*. 6th edition 2007, New York: W.H. Freeman and Company. 574.
8. Goh, F.G. and Midwood, K.S., *Intrinsic danger: activation of Toll-like receptors in rheumatoid arthritis.* Rheumatology, 2012. **51**(1): p. 7-23.
9. Huang, Q.Q. and Pope, R.M., *The role of toll-like receptors in rheumatoid arthritis.* Current rheumatology reports, 2009. **11**(5): p. 357-364.
10. Tran, C.N., et al., *Presentation of arthritogenic peptide to antigen-specific T cells by fibroblast-like synoviocytes.* Arthritis & Rheumatism, 2007. **56**(5): p. 1497-1506.
11. Choy, E., *Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis.* Rheumatology, 2012. **51**: p. V3-V11.
12. Lefevre, S., et al., *Synovial fibroblasts spread rheumatoid arthritis to unaffected joints.* Nature Medicine, 2009. **15**(12): p. 1414-U1410.
13. Schett, G., *Cells of the synovium in rheumatoid arthritis - Osteoclasts.* Arthritis Research & Therapy, 2007. **9**(1).
14. Masui, T., et al., *Expression of inflammatory cytokines, RANKL and OPG induced by titanium, cobalt-chromium and polyethylene particles.* Biomaterials, 2005. **26**(14): p. 1695-1702.
15. Kim, K.W., et al., *TLR-3 enhances osteoclastogenesis through upregulation of RANKL expression from fibroblast-like synoviocytes in patients with rheumatoid arthritis.* Immunology Letters, 2009. **124**(1): p. 9-17.
16. Boissier, M.C., *Cell and cytokine imbalances in rheumatoid synovitis.* Joint Bone Spine, 2011. **78**(3): p. 230-234.
17. Simsek, I., *TNF inhibitors - new and old agents for rheumatoid arthritis.* Bulletin of the NYU hospital for joint diseases, 2010. **68**(3): p. 204-210.
18. Schett, G. and Gravallese, E., *Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment.* Nature Reviews Rheumatology, 2012. **8**(11): p. 656-664.
19. Redlich, K. and Smolen, J.S., *Inflammatory bone loss: pathogenesis and therapeutic intervention.* Nature Reviews Drug Discovery, 2012. **11**(3): p. 234-250.
20. Harashima, S.I., Tsukamoto, H., and Horiuchi, T., *Osteoprotegerin and receptor activator of nuclear factor kappa B ligand expression in fibroblast-like synoviocytes from rheumatoid arthritis and osteoarthritis patients.* Rheumatology, 2004. **43**(3): p. 396-397.

21. Feuerherm, A.J., et al., *Elevated levels of osteoprotegerin (OPG) and hepatocyte growth factor (HGF) in rheumatoid arthritis.* Scandinavian Journal of Rheumatology, 2001. **30**(4): p. 229-234.
22. Kotake, S., et al., *Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients.* Arthritis & Rheumatism, 2001. **44**(5): p. 1003-1012.
23. Crotti, T.N., et al., *Receptor activator NF-kappaB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis.* Annals of the Rheumatic Diseases, 2002. **61**(12): p. 1047-1054.
24. Walsh, N.C. and Gravallese, E.M., *Bone remodeling in rheumatic disease: a question of balance.* Immunological reviews, 2010. **233**(1): p. 301-312.
25. Pettit, A.R., et al., *RANKL protein is expressed at the pannus-bone interface at sites of articular bone erosion in rheumatoid arthritis.* Rheumatology (Oxford), 2006. **45**(9): p. 1068-1076.
26. Diarra, D., et al., *Dickkopf-1 is a master regulator of joint remodeling.* Nature Medicine, 2007. **13**(2): p. 156-163.
27. R&D, S. *Dkk-1: Upsetting the Balance in Rheumatoid Arthritis*. 2007 [cited 2012 May 1]; Available from: http://www.rndsystems.com/cb_detail_objectname_su07_DKK1.aspx.
28. Hashimoto, C., Hudson, K.S., and Anderson, K.V., *The Toll gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein.* Cell, 1988. **52**(2): p. 269-279.
29. Philippe, L., et al., *TLR2 Expression Is Regulated by MicroRNA miR-19 in Rheumatoid Fibroblast-like Synoviocytes.* Journal of Immunology, 2012. **188**(1): p. 454-461.
30. Brentano, F., et al., *RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3.* Arthritis & Rheumatism, 2005. **52**(9): p. 2656-2665.
31. Hung, J., et al., *Toll-like Receptors and Innate Immunity*, in *Pathways* 2008, SABiosciences. p. 16.
32. Gnjatic, S., Sawhney, N.B., and Bhardwaj, N., *Toll-like receptor agonists: are they good adjuvants?* Cancer Journal, 2010. **16**(4): p. 382-391.
33. Kim, K.W., et al., *Human rheumatoid synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-2 and TLR-4 activation.* Immunology Letters, 2007. **110**(1): p. 54-64.
34. Ospelt, C., et al., *Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: Toll-like receptor expression in early and longstanding arthritis.* Arthritis & Rheumatism, 2008. **58**(12): p. 3684-3692.
35. Seibl, R., et al., *Expression and Regulation of Toll-Like Receptor 2 in Rheumatoid Arthritis Synovium.* The American Journal of Pathology, 2003. **162**(4): p. 1221-1227.
36. van der Heijden, I.M., et al., *Presence of bacterial DNA and bacterial peptidoglycans in joints of patients with rheumatoid arthritis and other arthritides.* Arthritis & Rheumatism, 2000. **43**(3): p. 593-598.

37. Stahl, H.D., et al., *Detection of multiple viral DNA species in synovial tissue and fluid of patients with early arthritis.* Annals of the Rheumatic Diseases, 2000. **59**(5): p. 342-346.
38. Schumacher Jr, H.R., et al., *Chlamydia trachomatis nucleic acids can be found in the synovium of some asymptomatic subjects.* Arthritis & Rheumatism, 1999. **42**(6): p. 1281-1284.
39. Pecchi, E., et al., *Prostaglandins and sickness behavior: Old story, new insights.* Physiology & Behavior, 2009. **97**(3-4): p. 279-292.
40. Pilbeam, C.C., et al., *Prostaglandins and Bone Metabolism*, in *Principles of Bone Biology (Third Edition)*, John, P.B., Lawrence, G.R., and Martin, T.J., Editors. 2008, Academic Press: San Diego. p. 1235-1271.
41. Wu, C.Y., et al., *TLR4-Dependent Induction of Vascular Adhesion Molecule-1 in Rheumatoid Arthritis Synovial Fibroblasts: Roles of Cytosolic Phospholipase A(2)alpha/Cyclooxygenase-2.* Journal of Cellular Physiology, 2010. **223**(2): p. 480-491.
42. Balsinde, J., et al., *Group V phospholipase A(2)-mediated oleic acid mobilization in lipopolysaccharide-stimulated P388D(1) macrophages.* Journal of Biological Chemistry, 2000. **275**(7): p. 4783-4786.
43. Jorgensen, K.M., et al., *Platelet activating factor stimulates arachidonic acid release in differentiated keratinocytes via arachidonyl non-selective phospholipase A2.* Archives of Dermatological Research, 2010. **302**(3): p. 221-227.
44. Carrillo, C., Cavia Mdel, M., and Alonso-Torre, S., *Role of oleic acid in immune system; mechanism of action; a review.* Nutrición Hospitalaria, 2012. **27**(4): p. 978-990.
45. Dietrich, J.W., Goodson, J.M., and Raisz, L.G., *Stimulation of bone resorption by various prostaglandins in organ culture.* Prostaglandins, 1975. **10**(2): p. 231-240.
46. Wani, M.R., et al., *Prostaglandin E-2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: Synergistic activation of differentiation, cell spreading, and fusion.* Endocrinology, 1999. **140**(4): p. 1927-1935.
47. Yoshida, K., et al., *Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(7): p. 4580-4585.
48. Fortier, I., et al., *Immunolocalization of the prostaglandin E-2 receptor subtypes in human bone tissue: differences in foetal, adult normal, osteoporotic and pagetic bone.* Prostaglandins Leukotrienes and Essential Fatty Acids, 2004. **70**(5): p. 431-439.
49. Kasugai, S., et al., *Expression of prostaglandin E receptor subtypes in bone: expression of EP2 in bone development.* Bone, 1995. **17**(1): p. 1-4.
50. Suda, K., et al., *Suppression of osteoprotegerin expression by prostaglandin E-2 is crucially involved in lipopolysaccharide-induced osteoclast formation.* Journal of Immunology, 2004. **172**(4): p. 2504-2510.
51. Burke, J.E. and Dennis, E.A., *Phospholipase A(2) Biochemistry.* Cardiovascular Drugs and Therapy, 2009. **23**(1): p. 49-59.
52. Magrioti, V. and Kokotos, G., *Phospholipase A(2) inhibitors as potential therapeutic agents for the treatment of inflammatory diseases.* Expert Opinion on Therapeutic Patents, 2010. **20**(1): p. 1-18.

53. Kramer, R.M., et al., *Structure and properties of a human non-pancreatic phospholipase A2.* Journal of Biological Chemistry, 1989. **264**(10): p. 5768-5775.
54. Hegen, M., et al., *Cytosolic phospholipase A(2)alpha-deficient mice are resistant to collagen-induced arthritis.* Journal of Experimental Medicine, 2003. **197**(10): p. 1297-1302.
55. Anthonsen, M.W., Solhaug, A., and Johansen, B., *Functional coupling between secretory and cytosolic phospholipase A2 modulates tumor necrosis factor-alpha- and interleukin-1beta-induced NF-kappa B activation.* Journal of Biological Chemistry, 2001. **276**(32): p. 30527-30536.
56. Anthonsen, M.W., et al., *Atypical lambda/iota PKC conveys 5-lipoxygenase/leukotriene B4-mediated cross-talk between phospholipase A2s regulating NF-kappa B activation in response to tumor necrosis factor-alpha and interleukin-1beta.* Journal of Biological Chemistry, 2001. **276**(38): p. 35344-35351.
57. Tai, N., et al., *Cytosolic phospholipase A2 alpha inhibitor, pyrroxyphene, displays anti-arthritic and anti-bone destructive action in a murine arthritis model.* Inflammation Research, 2010. **59**(1): p. 53-62.
58. Sommerfelt, R.M., Feuerherm, A.J. and Johansen, B., *Cytosolic phospholipase A2 regulates TNF-induced production of joint destructive effectors in fibroblast-like synoviocytes*, manuscript submitted to Scandinavian Journal of Rheumatology, 2013.
59. LGC/ATCC. *Product Description SW982*. 2012 [cited 2012 May 11]; Available from: http://www.lgcstandards-atcc.org/LGCAdvancedCatalogueSearch/ProductDescription/tabid/1068/Default.aspx.
60. McNearney, T.A., et al., *A peripheral neuroimmune link: glutamate agonists upregulate NMDA NR1 receptor mRNA and protein, vimentin, TNF-alpha, and RANTES in cultured human synoviocytes.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2010. **298**(3): p. R584-R598.
61. Lee, Y.S. and Choi, E.M., *Myricetin inhibits IL-1b-induced inflammatory mediators in SW982 human synovial sarcoma cells.* International Immunopharmacology, 2010. **10**(7): p. 812-814.
62. Yamazaki, T., et al., *Phenotypic characterization of a human synovial sarcoma cell line, SW982, and its response to dexamethasone.* In Vitro Cellular & Developmental Biology - Animal, 2003. **39**(8-9): p. 337-339.
63. Qiagen, *Critical Factors for Successful Real-Time PCR*, in *Sample and Assay Technologies* 2009, Qiagen.
64. Livak, K.J. and Schmittgen, T.D., *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-408.
65. Ramakers, C., et al., *Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data.* Neuroscience Letters, 2003. **339**(1): p. 62-66.
66. Pfaffl, M.W., Horgan, G.W., and Dempfle, L., *Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR.* Nucleic Acids Research, 2002. **30**(9): p. e36.

67. Lee, A.T.Y. and Pile, K., *Disease modifying drugs in adult rheumatoid arthritis*. 2003 [cited 2013 May 8]; Available from: http://www.australianprescriber.com/magazine/26/2/36/40 - .UYogUpVg2jI.
68. Fugelsnes, E., *Når kroppen angriper seg selv*. 2011 [cited 2012 May 31]; Available from: http://www.forskning.no/artikler/2011/mars/280850.
69. Huwiler, A., et al., *The ω3-polyunsaturated fatty acid derivatives AVX001 and AVX002 directly inhibit cytosolic phospholipase A2 and suppress PGE2 formation in mesangial cells.* British Journal of Pharmacology, 2012. **167**(8): p. 1691-1701.
70. Ludwig, J., et al., *Design and Synthesis of 1-Indol-1-yl-propan-2-ones as Inhibitors of Human Cytosolic Phospholipase A2alpha.* Journal of Medicinal Chemistry, 2006. **49**(8): p. 2611-2620.
71. Antonopoulou, G., et al., *Structure-activity relationships of natural and non-natural amino acid-based amide and 2-oxoamide inhibitors of human phospholipase A2 enzymes.* Bioorganic & Medicinal Chemistry, 2008. **16**(24): p. 10257-10269.
72. Snyder, D.W., et al., *Pharmacology of LY315920/S-5920, [[3-(aminooxoacetyl)-2-ethyl-1- (phenylmethyl)-1H-indol-4-yl]oxy] acetate, a potent and selective secretory phospholipase A2 inhibitor: A new class of anti-inflammatory drugs,* Journal of Pharmacology and Experimental Therapeutics, 1999. **288**(3): p. 1117-1124.
73. Lucas, K.K. and Dennis, E.A., *Distinguishing phospholipase A2 types in biological samples by employing group-specific assays in the presence of inhibitors.* Prostaglandins & Other Lipid Mediators, 2005. **77**(1-4): p. 235-248.
74. Ouellet, M. and M.D. Percival, *Effect of inhibitor time-dependency on selectivity towards cyclooxygenase isoforms.* Biochemical Journal, 1995. **306 (Pt 1)**: p. 247-251.
75. Qiagen, *RNeasy® Mini Handbook*, 2006, Qiagen.
76. Campbell, M. *cDNA Production*. 2002 [cited 2013 April 15]; Available from: http://www.bio.davidson.edu/courses/genomics/method/cdnaproduction.html.
77. Qiagen, *RT2 qPCR Primer Assay Handbook*, in *Sample & Assay Technologies* 2011, Qiagen. p. 30.
78. Lee, P.Y., et al., *Agarose gel electrophoresis for the separation of DNA fragments.* Journal of Visualized Experiments, 2012(62).
79. Berg, J.M., Tymoczko, J.L. and Stryer, L., *Biochemistry* 2002, New York: W H Freeman.
80. Sigma-Aldrich. *qPCR Technical Guide*. 2008 [cited 2013 April 4]; Available from: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General_Information/qpcr_technical_guide.Par.0001.File.tmp/qpcr_technical_guide.pdf.
81. O'Neill, L.A., *Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily.* Current Topics in Microbiology and Immunology, 2002. **270**: p. 47-61.
82. Nguyen, T., *LPS stimulation*, personal communication to Skuland, T., Trondheim 2013.
83. Inoue, H., et al., *Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts.* British Journal of Pharmacology, 2002. **136**(2): p. 287-295.

84. Cisar, L.A., Schimmel, R.J., and Mochan, E., *Interleukin-1 stimulation of arachidonic acid release from human synovial fibroblasts; blockade by inhibitors of protein kinases and protein synthesis.* Cellular Signalling, 1991. **3**(3): p. 189-199.
85. Harigai, M., et al., *Interleukin 1 and tumor necrosis factor-alpha synergistically increase the production of interleukin 6 in human synovial fibroblast.* Journal of Clinical & Laboratory Immunology, 1991. **34**(3): p. 107-113.
86. Sommerfelt, R.M., *TNF-induced IL-6 response*, personal communication to Skuland, T., Trondheim 2013.
87. Coller, H.A., Sang, L., and Roberts, J.M., *A new description of cellular quiescence.* PLOS Biology, 2006. **4**(3): p. e83.
88. Chen, B.R., et al., *Quiescent Fibroblasts Are More Active in Mounting Robust Inflammatory Responses Than Proliferative Fibroblasts.* PLOS ONE, 2012. **7**(11): p. e49232.
89. Tunyogi-Csapo, M., et al., *Cytokine-controlled RANKL and osteoprotegerin expression by human and mouse synovial fibroblasts: Fibroblast-mediated pathologic bone resorption.* Arthritis & Rheumatism, 2008. **58**(8): p. 2397-2408.
90. Meylan, E., et al., *RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation.* Nature Immunology, 2004. **5**(5): p. 503-507.
91. Schoppet, M., et al., *Osteoprotegerin expression in dendritic cells increases with maturation and is NF-kappa B-dependent.* Journal of Cellular Biochemistry, 2007. **100**(6): p. 1430-1439.
92. SABiosciences. *Search Result: NF-kappaB Binding Sites in DKK1 gene Promoter*. 2013 [cited 2013 May 10]; Available from: http://www.sabiosciences.com/chipqpcrsearch.php?gene=DKK1&species_id=0&factor=NF-kappaB&ninfo=n&ngene=n&nfactor=n.
93. Kishore, N., et al., *A Selective IKK-2 Inhibitor Blocks NF-κB-dependent Gene Expression in Interleukin-1β-stimulated Synovial Fibroblasts.* Journal of Biological Chemistry, 2003. **278**(35): p. 32861-32871.
94. Emery, J.G., et al., *Osteoprotegerin Is a Receptor for the Cytotoxic Ligand TRAIL.* Journal of Biological Chemistry, 1998. **273**(23): p. 14363-14367.
95. Miyashita, T., et al., *Osteoprotegerin (OPG) acts as an endogenous decoy receptor in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis of fibroblast-like synovial cells.* Clinical & Experimental Immunology, 2004. **137**(2): p. 430-436.
96. Morel, J., Audo, R., and Combe, B., *IL-1 but not IL-18 induces osteoprotegerin and TRAIL in rheumatoid arthritis synovial fibroblasts.* Arthritis Research & Therapy, 2003. **5**(Suppl 3): p. 66.
97. Weng, L.H., et al., *Dkk-1 promotes angiogenic responses and cartilage matrix proteinase secretion in synovial fibroblasts from osteoarthritic joints.* Arthritis & Rheumatism, 2012. **64**(10): p. 3267-3277.
98. Hardy, R., et al., *Synovial DKK1 expression is regulated by local glucocorticoid metabolism in inflammatory arthritis.* Arthritis Research & Therapy, 2012. **14**(5): p. R226.
99. Brändström, H., et al., *Regulation of Osteoprotegerin mRNA Levels by Prostaglandin E2 in Human Bone Marrow Stroma Cells.* Biochemical and Biophysical Research Communications, 1998. **247**(2): p. 338-341.

100. Bonewald, L.F. and Johnson, M.L, *Osteocytes, mechanosensing and Wnt signaling.* Bone, 2008. **42**(4): p. 606-615.
101. Sack, U., et al., *Interleukin-6 in synovial fluid is closely associated with chronic synovitis in rheumatoid arthritis.* Rheumatology International, 1993. **13**(2): p. 45-51.
102. Siegle, I., et al., *Expression of cyclooxygenase 1 and cyclooxygenase 2 in human synovial tissue: differential elevation of cyclooxygenase 2 in inflammatory joint diseases.* Arthritis & Rheumatism, 1998. **41**(1): p. 122-129.
103. Into, T., et al., *Effect of the antimicrobial peptide LL-37 on Toll-like receptors 2-, 3- and 4-triggered expression of IL-6, IL-8 and CXCL10 in human gingival fibroblasts.* Cellular Immunology, 2010. **264**(1): p. 104-109.
104. Matsumoto, C., et al., *Toll-like receptor 2 heterodimers, TLR2/6 and TLR2/1 induce prostaglandin E production by osteoblasts, osteoclast formation and inflammatory periodontitis.* Biochemical and Biophysical Research Communications, 2012. **428**(1): p. 110-115.
105. Lundberg, A.M., et al., *Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems.* Blood, 2007. **110**(9): p. 3245-3252.
106. Ruiperez, V., et al., *Coordinate Regulation of TLR-Mediated Arachidonic Acid Mobilization in Macrophages by Group IVA and Group V Phospholipase A(2)S.* Journal of Immunology, 2009. **182**(6): p. 3877-3883.
107. Balsinde, J. and Dennis, E.A., *Bromoenol Lactone Inhibits Magnesium-dependent Phosphatidate Phosphohydrolase and Blocks Triacylglycerol Biosynthesis in Mouse P388D1 Macrophages.* Journal of Biological Chemistry, 1996. **271**(50): p. 31937-31941.
108. Grkovich, A., et al., *TLR-4 mediated group IVA phospholipase A2 activation is phosphatidic acid phosphohydrolase 1 and protein kinase C dependent.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2009. **1791**(10): p. 975-982.
109. Kubota, A., et al., *Tumor necrosis factor-alpha promotes the expression of osteoprotegerin in rheumatoid synovial fibroblasts.* Journal of Rheumatology, 2004. **31**(3): p. 426-435.
110. Ziolkowska, M., et al., *High levels of osteoprotegerin and soluble receptor activator of nuclear factor kappa B ligand in serum of rheumatoid arthritis patients and their normalization after anti-tumor necrosis factor alpha treatment.* Arthritis & Rheumatism, 2002. **46**(7): p. 1744-1753.
111. Kim, Y.S., et al., *Effect of Cytosolic Phospholipase A(2) on Proinflammatory Cytokine-induced Bone Resorptive Genes Including Receptor Activator of Nuclear Factor Kappa B Ligand in Human Dental Pulp Cells.* Journal of Endodontics, 2010. **36**(4): p. 636-641.
112. Davanian, H., et al., *Signaling pathways involved in the regulation of TNFα-induced toll-like receptor 2 expression in human gingival fibroblasts.* Cytokine, 2012. **57**(3): p. 406-416.
113. Astashkina, A., Mann, B., and Grainger, D.W., *A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity.* Pharmacology & Therapeutics, 2012. **134**(1): p. 82-106.
114. El-Ali, J., Sorger, P.K., and Jensen, K.F., *Cells on chips.* Nature, 2006. **442**(7101): p. 403-411.
115. Lindstrom, S. and Andersson-Svahn, H., *Overview of single-cell analyses: microdevices and applications.* Lab on a Chip, 2010. **10**(24): p. 3363-3372.

Appendix

A. All biological replicas for the representative experiment in figure 3.3.1

Mean OPG mRNA fold changes and ±SEMs (n = 2):

Table A.1 Mean OPG fold changes after TLR agonists and IL-1β treatment for 6 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 6h	OPG fold change	SEM	
		Lower	Upper
FSL-1	6.4 *	3.2	18.6
P3C	4.8 *	2.2	12.8
PIC	2.3	1.1	9.1
IL-1β	6.7 *	3.8	22.8

Table A.2 Mean OPG fold changes after TLR agonists and IL-1β treatment for 24 hours.

Treatment, 24h	OPG fold change	SEM	
		Lower	Upper
FSL-1	1.7	0.4	14.5
P3C	1.2	0.3	10.4
PIC	0.7	0.2	5.9
IL-1β	1.7	0.5	12.8

Mean DKK1 mRNA fold changes and ±SEMs (n = 2):

Table A.3 Mean DKK1 fold changes after TLR agonists and IL-1β treatment for 6 hours.

Treatment, 6h	DKK1 fold change	SEM	
		Lower	Upper
FSL-1	2.6	1.5	5.2
P3C	1.4	0.6	4.6
PIC	1.2	0.7	2.3
IL-1β	2.8	1.7	5.7

Table A.4 Mean DKK1 fold changes after TLR agonists and IL-1β treatment for 24 hours.

Treatment, 24h	DKK1 fold change	SEM	
		Lower	Upper
FSL-1	1.5	0.7	4.2
P3C	1.9	1.0	6.4
PIC	1.1	0.5	2.8
IL-1β	1.4	0.7	4.5

B. Data for time curves in figure 3.3.2

Mean OPG mRNA fold changes and ±SEMs (n = 2, except for IL-1 where n = 1):

Table B.1 Mean OPG fold changes after TLR agonists and IL-1β treatment for 3 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 3h	OPG fold change	SEM	
		Lower	Upper
FSL-1	4.5 *	3.7	5.7
P3C	2.5	2.0	3.1
PIC	2.2 *	1.8	2.8
IL-1β	5.2	-	-

Table B.2 Mean OPG fold changes after TLR agonists and IL-1β treatment for 6 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 6h	OPG fold change	SEM	
		Lower	Upper
FSL-1	4.6	3.7	5.7
P3C	3.2 *	2.0	3.1
PIC	2.4	1.8	2.8
IL-1β	5.0	-	-

Table B.3 Mean OPG fold changes after TLR agonists and IL-1β treatment for 9 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 9h	OPG fold change	SEM	
		Lower	Upper
FSL-1	3.6 *	2.8	4.6
P3C	3.3 *	2.8	4.0
PIC	2.6 *	2.2	3.1
IL-1β	4.3	-	-

Table B.4 Mean OPG fold changes after TLR agonists and IL-1β treatment for 12 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 12h	OPG fold change	SEM	
		Lower	Upper
FSL-1	2.4	2.0	3.0
P3C	3.0	2.6	3.4
PIC	2.1 *	1.9	2.4
IL-1β	2.4	-	-

Mean DKK1 fold changes and ±SEMs (n = 2, except for IL-1 where n = 1):

Table B.5 Mean DKK1 fold changes after TLR agonists and IL-1β treatment for 3 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 3h	DKK1 fold change	SEM	
		Lower	Upper
FSL-1	0.3 *	0.3	0.4
P3C	0.5	0.4	0.6
PIC	0.3 *	0.2	0.3
IL-1β	0.4	-	-

Table B.6 Mean DKK1 fold changes after TLR agonists and IL-1β treatment for 6 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 6h	DKK1 fold change	SEM	
		Lower	Upper
FSL-1	2.7	1.9	3.7
P3C	1.1	0.8	1.4
PIC	2.5 *	2.4	2.7
IL-1β	4.8	-	-

Table B.7 Mean DKK1 old changes after TLR agonists and IL-1β treatment for 9 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 9h	DKK1 fold change	SEM	
		Lower	Upper
FSL-1	2.3 *	2.0	2.7
P3C	2.1 *	2.0	2.1
PIC	4.0 *	3.8	4.1
IL-1β	2.5	-	-

Table B.8 Mean DKK1 fold changes after TLR agonists and IL-1β treatment for 12 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 12h	DKK1 fold change	SEM	
		Lower	Upper
FSL-1	1.7 *	1.7	1.8
P3C	1.9 *	1.9	2.0
PIC	4.8 *	4.2	5.4
IL-1β	1.6	-	-

C. All biological replicas for the representative experiment in figure 3.5.1

Mean OPG mRNA fold changes and ±SEMs (n = 5):

Table C.1 Mean OPG fold changes after 9 hours stimulation with the TLR2/6 agonist FSL-1 or FSL-1 plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment, 9h	OPG fold change	SEM	
		Lower	Upper
FSL-1	3.5 *	2.0	6.4
FSL-1 + AVX002	3.7	1.9	8.1
FSL-1 + InhibX	3.5	1.7	8.3
FSL-1 + Indomethacin	2.8	1.5	5.7

Table C.2 Mean OPG fold changes after 9 hours stimulation with the TLR1/2 agonist P3C or P3C plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1, and the number sign symbolizes significant difference from the TLR agonist alone ($p < 0.05$).

Treatment, 9h	OPG fold change	SEM	
		Lower	Upper
P3C	3.6 *	2.2	6.0
P3C + AVX002	2.6	1.4	4.2
P3C + InhibX	2.1	1.2	4.5
P3C + Indomethacin	2.1 #	1.2	3.4

D. All biological replicas for the representative experiment in figure 3.6.1

Mean DKK1 mRNA fold changes and ±SEMs (n = 5):

Table D.1 Mean DKK1 fold changes after 9 hours stimulation with the TLR2/6 agonist FSL-1 or FSL-1 plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The number sign symbolizes significant difference from the TLR agonist alone ($p < 0.05$).

Treatment, 9h	DKK1 fold change	SEM	
		Lower	Upper
FSL-1	2.3	0.9	5.5
FSL-1 + AVX002	2.5	0.9	7.3
FSL-1 + InhibX	2.0	0.7	5.5
FSL-1 + Indomethacin	1.0 #	0.3	2.5

Table D.2 Mean DKK1 fold changes after 9 hours stimulation with the TLR1/2 agonist P3C or P3C plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1, and the number sign symbolizes significant difference from the TLR agonist alone ($p < 0.05$).

Treatment, 9h	DKK1 fold change	SEM	
		Lower	Upper
P3C	3.2 *	1.1	7.7
P3C + AVX002	1.5 #	0.6	3.5
P3C + InhibX	1.4 #	0.5	3.4
P3C + Indomethacin	0.9 #	0.3	2.3

E. All biological replicas for the representative experiment in figure 3.7.1

Mean IL-6 mRNA fold changes and ±SEMs (n = 5):

Table E.1 Mean IL-6 fold changes after 9 hours stimulation with the TLR2/6 agonist FSL-1 or FSL-1 plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1, and the number sign symbolizes significant difference from the TLR agonist alone ($p < 0.05$).

Treatment, 9h	IL-6 fold change	SEM	
		Lower	Upper
FSL-1	84.0 *	54.5	123.2
FSL-1 + AVX002	81.6	50.4	125.1
FSL-1 + InhibX	99.6	51.6	248.5
FSL-1 + Indomethacin	38.3 #	25.9	59.3

Table E.2 Mean IL-6 fold changes after 9 hours stimulation with the TLR1/2 agonist P3C or P3C plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1, and the number sign symbolizes significant difference from the TLR agonist alone ($p < 0.05$).

Treatment, 9h	IL-6 fold change	SEM	
		Lower	Upper
P3C	48.0 *	28.7	75.2
P3C + AVX002	22.3 #	13.6	34.9
P3C + InhibX	20.6 #	13.8	33.9
P3C + Indomethacin	15.3 #	9.2	25.0

F. All biological replicas for the representative experiment in figure 3.8.1

Mean COX-2 mRNA fold changes and ±SEMs (n = 5):

Table F.1 Mean COX-2 fold changes after 9 hours stimulation with the TLR2/6 agonist FSL-1 or FSL-1 plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1, and the number sign symbolizes significant difference from the TLR agonist alone ($p < 0.05$).

Treatment, 9h	COX-2 fold change	SEM	
		Lower	Upper
FSL-1	29.9 *	14.8	64.2
FSL-1 + AVX002	20.4	9.8	41.4
FSL-1 + InhibX	23.5	12.4	55.1
FSL-1 + Indomethacin	15.3 #	8.5	33.3

Table F.2 Mean COX-2 fold changes after 9 hours stimulation with the TLR1/2 agonist P3C or P3C plus a $cPLA_2$ inhibitor (AVX002 or InhibX) or a COX inhibitor (Indomethacin). The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1, and the number sign symbolizes significant difference from the TLR agonist alone ($p < 0.05$).

Treatment, 9h	COX-2 fold change	SEM	
		Lower	Upper
P3C	14.4 *	7.1	32.3
P3C + AVX002	4.8 #	2.0	12.2
P3C + InhibX	4.6 #	2.6	9.4
P3C + Indomethacin	5.9 #	3.1	12.3

G. Data for time curves in figure 3.9.1

Mean AA release fold changes and ±SDs (n = 3):

Table G.1 Mean AA release fold changes after TLR agonists and IL-1β treatment for 3, 6, 9, 12 and 24 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

Treatment	3h	6h	9h	12h	24h
FSL-1 100 ng/ml	1.8	3.9 *	4.4 *	3.9 *	2.6
±SD	0.9	1.5	1.3	0.7	0.5
FSL-1 50 ng/ml	1.7	4.2 *	4.1 *	4.2 *	2.6
±SD	0.6	1.6	1.2	0.8	0.5
P3C 300 ng/ml	1.1	2.4	3.0 *	2.9 *	1.9
±SD	0.2	1.0	1.1	0.5	0.3
P3C 100 ng/ml	1.0	1.9	2.3	2.3	1.7
±SD	0.2	1.0	0.9	0.6	0.3
PIC 5 µg/ml	1.1	2.0 *	2.5 *	2.9 *	2.2 *
±SD	0.4	0.3	0.4	0.5	0.3
PIC 1 µg/ml	1.0	1.9 *	2.3 *	2.9 *	2.2 *
±SD	0.3	0.3	0.5	0.5	0.3
IL-1β 10 ng/ml	1.5	3.8 *	4.5 *	4.4 *	2.9
±SD	0.4	1.8	1.6	0.8	0.6

H. All biological replicas for the representative experiment in figure 3.11.1

Mean RANKL mRNA fold changes and ±SEMs (n = 3):

Table H.1 Mean RANKL fold changes after TNF−α treatment for 3, 6, 12 and 24 hours.

TNF-α treatment	RANKL fold change	SEM	
		Lower	Upper
6h	1.2	0.3	6.6
12h	1.0	0.3	3.8
24h	0.9	0.4	3.3
48h	0.9	0.3	3.1

Mean DKK1 mRNA fold changes and ±SEMs (n = 3):

Table H.2 Mean DKK1 fold changes after TNF−α treatment for 3, 6, 12 and 24 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

TNF-α treatment	DKK1 fold change	SEM	
		Lower	Upper
6h	2.6 *	1.2	5.9
12h	1.4	0.8	2.0
24h	1.6	0.9	2.6
48h	1.3	0.8	2.2

I. All biological replicas for the representative experiment in figure 3.12.1

Mean TLR mRNA fold changes and ±SEMs (n = 3):

Table I.1 Mean TLR1 fold changes after TNF−α treatment for 3, 6, 12 and 24 hours.

TNF-α treatment	TLR1 fold change	SEM	
		Lower	Upper
6h	1.6	0.6	6.7
12h	1.1	0.4	3.9
24h	1.2	0.3	8.9
48h	0.7	0.1	3.2

Table I.2 Mean TLR2 fold changes after TNF−α treatment for 3, 6, 12 and 24 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

TNF-α treatment	TLR2 fold change	SEM	
		Lower	Upper
6h	5.7 *	3.9	8.5
12h	5.8 *	4.6	7.1
24h	3.2 *	2.6	3.9
48h	2.5	1.4	4.9

Table I.3 Mean TLR3 fold changes after TNF−α treatment for 3, 6, 12 and 24 hours. The asterisk denotes significant difference from the control ($p < 0.05$), which is set to 1.

TNF-α treatment	TLR3 fold change	SEM	
		Lower	Upper
6h	3.1 *	2.6	3.8
12h	2.2	1.5	3.0
24h	1.8 *	1.6	2.0
48h	0.8	0.7	0.9

J. All biological replicas for the representative experiment in figure 3.13.1

Mean IL-6 mRNA fold change and ±SEM (n = 3):

Table J.1 Mean IL-6 fold change after TNF−α treatment for 24 hours.

TNF treatment	IL-6 fold change	SEM	
		Lower	Upper
24h	12.4	4.5	47.3