Randi Magnus Sommerfelt

Molecular mechanisms of inflammation – a central role for cytosolic phospholipase A2

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Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biology

NTNU – Trondheim
Norwegian University of
Science and Technology

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Summary

Bioactive lipids are central in regulating the inflammatory process and imbalance in lipid mediator signaling contributes to progression of pathological conditions such as atherosclerosis, allergy, autoimmunity, degenerative diseases and cancer. Phospholipase A2 (PLA2) enzymes release fatty acids such as arachidonic acid (AA) and a lysophospholipid from cellular membranes. Lysophospholipids can me metabolized to biologically active lipid mediators including platelet-activating factor (PAF). PAF is a potent mediator of inflammation, but can also exert a range of other physiological and pathophysiological processes including apoptosis, proliferation and cancer development. AA is a precursor of many bioactive lipid including prostaglandins such as prostaglandin E2 (PGE2), a potent immunoregulator and inducer of inflammation, fever and pain.

In particular cytosolic phosholipase A2 (cPLA2α) is associated with inflammation and inflammatory disease as a main enzyme mediating AA release and proinflammatory eicosanoid production, and is proposed as a future therapeutic target. However, lipid signaling is complex and sophisticatedly regulated, and the downstream consequences of cPLA2α inhibition are not fully understood. The overall objective of this thesis was to investigate the role of PLA2 enzymes, in particular cPLA2α, and downstream lipid messengers in cellular signaling mechanisms involved in chronic inflammatory disease. In Paper I, we investigated the role of PAF in differentiated keratinocytes, a cellular model system for psoriatic skin. We found that PAF did not primarily induce pro-inflammatory signaling, but rather proliferative responses possibly linking the inflammatory response to re-epithelialization and wound-healing. In the second part of this thesis comprising Papers II-IV, we focused on the role of cPLA2α in regulating pro-inflammatory signaling pathways central in the pathogenesis of rheumatoid arthritis (RA). In Paper II, we found cPLA2α to regulate joint-destructive and pro-inflammatory effectors induced by tumor necrosis factor (TNF), a "master" cytokine in RA. In Papers III and IV, we investigated the role of cPLA2α in modulating TLR-induced signaling. TLRs constitute a central part in the innate immune system sensing invading pathogens and tissue injury. However, TLRs can also induce "sterile" inflammation by recognizing molecules derived from the host itself, and increased TLR

activation is believed to contribute to the pathogenesis of a range of inflammatory and autoimmune diseases including RA. We found that cPLA2α regulates TLR-induced activation of the transcription factor NF-κB and expression of several pro-inflammatory mediators. We furthermore identified PGE2 and possibly other related prostanoids as actors in this mechanism.

Taken together, our findings expand the understanding of cPLA2α as a central regulator of molecular mechanisms in chronic inflammation, and enlighten the potential role of cPLA2α and PAF in linking the inflammatory and proliferatory processes.

List of abbreviations

AA	Arachidonic acid
AKT	Protein kinase B
AP-1	Activator protein-1
CIA	Collagen-induced arthritis
CIP	Ceramide-1-phosphate
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
CYPP450	Cytochrome P-450
cPLA2	Cytosolic phospholipase A2
cAMP	Cyclic adenosine monophosphate
DAG	Diacylglycerol
DAMP	Damage accociated molecular patterns
DHA	Docosahexaenoic acid
DNA	Deoxuribonuclease
ds	Double-stranded
EGF	Epidermal growth factor
EGFR	EGF receptor
EP	Prostaglandin E2 receptor
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinases
FLS	Fibroblast-like synoviocytes
FSL-1	(S,R)-(2,3-bispalitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe
GPCR	G-protein coupled receptor
IFN	Interferon
IG	Immunoglobulin
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukine
IL1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist

IL-6R	IL-6 receptor
IP3	Inositol triphosphate
iPLA2	Calsium-independent phospholipase A2
IRAK	IL-1 receptor-associated kinases
IRF	IFN regulatory factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
LOX	Lipoxygenase
LPS	Lipopolysaccaride
LT	Leukotriene
LPX	Lipooxygenase
LX	Lipoxane
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MKK	MAP kinase kinase
MMP	Matrix metalloproteinase
MyD88	Myeloid differnentiation factor 88
NF-κB	Nuclear factor-κB
NLR	Nucleotide-binding oligomerization-domain protein-like receptors
NSAID	Nonsteroidal anti-inflammatory drug
Pam$_3$CSK$_4$	S-[2,3Bis(palmitoyloxi)-(2-RS)-propyl]-N-palmitoyl-®-Cys-(S)-Ser-(S)Lys$_4$-OH
PAMP	Pattern accociated molecular patterns
PI3K	Phosphatidylinositol 3-kinase
PAF	Platelet activating factor
PAF-AH	PAF acetyl hydrolase
PAFR	PAF receptor
PG	Prostaglandin
PGI	Prostacyclin
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2

PLC	Phospholipase C
PLD	Phospholipase D
PPAR	Nuclear peroxisome proliferator activated receptors
PPR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor B ligand
RNA	Ribonucleic acid
S1P	Sphingosine-1-phosphate
sPLA2	Secretory phospholipase A2
ss	Single-stranded
STAT	Signal transducer and activator of transcription
TAK	Transforming growth factor β-activated kinase
TGBβ	Transforming growth factor β
TIR	Toll/interleukin receptor
TIRAP	MyD88 adaptor-like
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain containing adaptor inducing IFNβ
TX	Thromboxane

List of Papers

Paper I:

Feuerherm, A. J., Jørgensen, K. M., <u>Sommerfelt, R. M</u>., Eidem, L., Lægreid, A., Johansen, B. **Platelet-activating factor induced proliferation in differentiated keratinocytes.** (2013) *Mol Cell Biochem* 384, pp83-94.

Paper II:

<u>Sommerfelt, R. M.</u>[*], Feuerherm, A. J.[*], Jones, K., Johansen, B. **Cytosolic phospholipase A2 regulates TNF-induced production of joint destructive effectors in synoviocytes**. (2013) *PLOS One* 8, e83555 [*]Shared authorship

Paper III:

<u>Sommerfelt, R. M.</u>[*], Feuerherm, A. J.[*], Skuland, T., Johansen, B. **Cytosolic phospholipase A2 modulates TLR2 signaling in synoviocytes.** *(Manuscript)* [*]Shared authorship

Paper IV:

<u>Sommerfelt, R. M.</u>, Feuerherm, A. J., Nguyen, T., Johansen, B. **cPLA2α regulates TLR2/6-induced NF-κB activation and IL-6 production through COX/PGE$_2$ in human synoviocytes.** *(Manuscript)*

Declaration of contribution:

Paper I: I performed all qPCR analyses and contributed significantly to figure making of these results. I contributed less to the final writing of the manuscript.

Paper II: I contributed significantly to formulation of the scientific problem, to the planning of experiments, generating experimental material and performing the experiments, to data analysis and writing of this manuscript.

Paper III: I contributed significantly to formulation of the scientific problem, to the planning of experiments, generating experimental material and performing the experiments, to data analysis and writing of this manuscript.

Paper IV: I contributed significantly to formulation of the scientific problem, to the planning of experiments, generating experimental material and performing the experiments, to data analysis and writing of this manuscript.

1. Introduction

The field of translational medicine can be described as an effort to carry scientific discoveries and knowledge from "bench to bedside". Basic research advances, such as studies of biological processes in cell cultures or animal models, provides an understanding of fundamental mechanisms in disease and can be built on to develop novel therapies or pinpoint new therapeutic targets. In this thesis, molecular mechanisms in inflammation are investigated in cellular model systems, in order to expand our understanding of dysregulated signaling processes in chronic inflammatory disease. From a translational research point of view, these results can contribute in the development of a new therapeutic approach in treating chronic inflammatory diseases.

1.1. Inflammation

The inflammatory response is part of our natural host-defense system and descriptions of its characteristics date back to ancient Egyptian and Greek cultures. More than 2000 years ago the roman doctor Aulus Celsius described the four hallmarks of inflammation; *rubor* (redness), *tumor* (swelling), *calor* (heat) and *dolor* (pain). Two hundred years later, Galen recognized inflammation as a reaction of the body against injury and added a fifth characteristic, *functio laesa,* loss of function, as pictured in Figure 1 [1]. During the 20th century a rapid advancement in the understanding of molecular function and mechanisms underlying inflammation took place, and our understanding of the inflammatory process is still expanding. Acute inflammation is a beneficial host defense mechanism protecting the body from infection and other insults, and our health entirely relies on its well-functioning. The Scottish surgeon John Hunter wrote in 1794 that "Inflammation in itself is not to be considered as a disease but as a salutary operation, consequent either to some violence or some disease" [2]. This insight emphasize that the inflammatory response is essential to remain healthy and maintain homeostasis. When it occurs, it is normally well regulated, self-limiting and resolves rapidly thus avoiding excessive damage to the host. However, the outcome of the inflammatory process is not always successful resolution and repair of damaged tissue. Chronic inflammatory diseases develop as a result of genetic and environmental interplay which

is still only partially understood and represent a major challenge. There has been a drastic increase in the prevalence of chronic inflammatory diseases since the end of World War II, particularly in the western world, possibly due to genetic and epigenetic factors in combinations with changes in diet and lifestyle [3, 4]. To understand how the highly complex and sophisticated signaling circuits are regulated in inflammation and chronic inflammatory disease is of crucial importance when it comes to discovering and developing new therapeutic strategies.
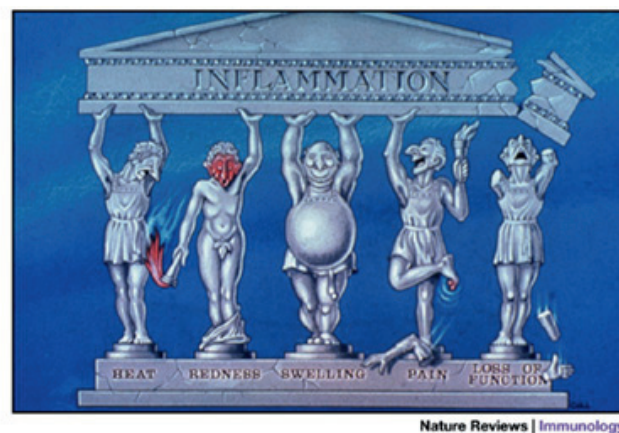
Figure 1: Inflammation depicted by five Greeks representing the cardinal signs of inflammation. Heat, Redness, Swelling, Pain and Loss of function – first described by Celcius more than two hundred years ago, and as relevant today as they were then. From Lawrence *et al.* 2002, with permission [5].

1.1.1. The course of inflammation

Inflammation involves interactions between many different cell types, and production of and response to numerous chemical signaling mediators. Our immune system is divided into two general types; the adaptive immune system and the innate immune system. The innate immune system constitutes a first-line defense in turn activating the adaptive immune system; a more specific, but slower defense mechanism evolving throughout our life. By rearrangement of the immunoglobulin genes and development of pathogen-specific antigens, immune cells develop immunological memory. Specific pathogens and infected cells can thereby be recognized and destructed. The innate (non-specific) immune system has no antigenic memory and is not affected by previous exposure to

infectious agents. The innate immune response is triggered by entrance of "foreign material" such as pathogens and allergens, tissue damage or non-pathogenic materials and is initiated by increased blood-flow to the affected area. A cascade of events is then effectuated including production of a variety of inflammatory mediators including cytokines, vasoactive mediators and lipid mediators such as eicosanoids. The main and most immediate effect of these mediators is that leukocytes, normally restricted to the blood-flow, is recruited and gain access to the extravascular tissue at the site of action through increased capillary permeability [6]. Further production of inflammatory mediators cause continuant recruitment of immune cells and perpetuate the inflammatory response until resolution signals are effectuated [7]. This acute inflammatory response can be triggered within minutes, and may persist for several days. Negative feed-back mechanisms such as inhibition of pro-inflammatory signaling cascades, production of anti-inflammatory or pro-resolving cytokines and lipid mediators, shedding of receptors for inflammatory molecules and activation of regulatory cells are central in resolution of inflammation [8, 9].

1.1.2. Inflammation and wound healing

In response to tissue injury, various intercellular and intracellular processes are activated and coordinated in order to restore tissue integrity and homeostasis [10]. The first stage of wound healing is the acute inflammatory response acting to prevent blood and fluid loss through the coagulation cascade, to remove dead or dying tissues and to prevent infection. The second stage occurring 2-10 days after wounding is characterized by new tissue formation, and involves cellular proliferation and migration of various cell types including keratinocytes and fibroblasts, and angiogenesis. During the third stage – tissue remodeling – feed-back loops terminate all activated processes and homeostasis is restored; a process which can last for a year or more [10]. Lipid mediators including eicosanoids, endocannabinoids and sphingolipids play critical roles in regulating wound healing and tissue repair through modulation of the inflammatory process [11]. Interestingly, microarray studies have revealed that in healing skin wounds, the gene expression patterns strongly resembles that of highly malignant

tumors [12], emphasizing the link between the inflammatory process, proliferation and cancer development.

1.1.2. Cytokines and cytokine receptors

Cytokines are a large family of relatively small, secreted non-enzymatic peptide hormones including chemokines, interferons, interleukins and tumor necrosis factors [13]. Through specific receptors, cytokines elicit a range of physiological effects including regulation of inflammation and immune responses. The cytokine network is normally self-regulating, balancing the expression of soluble receptors, receptor antagonists and antibodies to cytokines. Dysregulation of the equilibrium between pro- and anti-inflammatory cytokines is a driving force in pathologic inflammation (Figure 2). Interleukin (IL)-1β, IL-6, and TNF are central and pleiotropic effectors in the inflammatory response, and will be briefly described below focusing on their role in human disease.
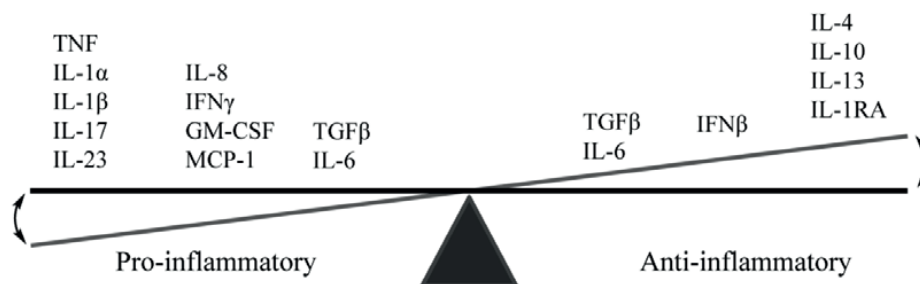
Figure 2: Cytokine balance in inflammatory disease. In chronic inflammatory disease, the normally tightly regulated balance between pro- and anti-inflammatory cytokines is skewed. IL-6 and TGFβ are placed at both sides of the fulcrum, as they can elicit both pro- and anti-inflammatory effects depending on stimuli, cell type and tissue. IFN-interferon; GM-CSF-granulocyte–macrophage colony-stimulating factor; MCP-1-monocyte chemotactic protein-1; TGBβ- transforming growth factor β; IL-1RA-interleukin-1 receptor antagonist. Modified from Arend 2001, with permission [14].

IL-1β is a member of the IL-1 superfamily and is central in innate immune responses and the pathogenesis of a range of inflammatory disorders. An inactive IL-1β precursor (proIL-1β) is produced by transcriptional regulation upon pro-inflammatory

stimuli including TNF or IL-1β itself [15]. Active IL-1β is produced by cleavage of proIL-1β by caspase-1 activity. Caspase-1 is activated by a protein complex called the inflammasome, which plays a crucial role in IL-1β production and is implicated in several inflammatory and autoimmune disorders [16]. There are two IL-1β receptors; IL-1R1 is the main receptor mediating cellular signaling cascades, whereas IL-1R2 may act as a decoy receptor suppressing IL-1β signaling [17]. The IL-1R family contains a cytoplasmic Toll/IL-1 receptor (TIR) domain [18], thus sharing commonalities with the toll-like receptor (TLR) signaling pathways (TLR signaling will be described below). IL-1 receptor antagonist (IL-1Ra) is a naturally produced inhibitor of IL-1β signaling, and imbalance between IL-1Ra and IL-1β is implicated in a number of diseases [19]. The central role of IL-1β in human disease is emphasized by the multiple clinical trials where IL-1β-blocking biologics (including receptor antagonists, soluble decoy receptors, and monoclonal anti-interleukin-1β antibodies) are shown effective in ameliorating a variety of inflammatory diseases such as rheumatoid arthritis (RA), type II diabetes and Behcet's disease [20].

IL-6 is a pleiotropic actor in the immune response, and is associated with several autoimmune, chronic inflammatory and proliferative diseases including multiple sclerosis, RA and cancer [21]. The IL-6 receptor (IL-6R) is expressed in two forms, soluble (sIL-6R) and membrane-bound. Upon ligand binding, the IL-6R signals through the JAK1–STAT3, RAS–MAPK, and PI3K–AKT pathways to induce transcription of genes involved in physiological, pro-inflammatory and anti-inflammatory processes including survival, proliferation, differentiation, osteogenesis/osteolysis, angiogenesis, and immune modulation [22]. Clinical trials on biologics targeting various factors in IL-6 signaling (including IL-6, IL-6R, sIL-6R, JAK, and STAT3) have shown promising effects in diseases including RA, systemic lupus and multiple myeloma [22].

TNF is extensively studied over the last 4 decades and is important in diverse cellular processes such as septic shock, induction of other cytokines, cell proliferation, differentiation, necrosis and apoptosis. The two TNF receptors TNFR1 and TNFR2 can be membrane bound or soluble [23]. TNFR1 is constitutively expressed in most tissues, while TNFR2 is inducible and typically found in cells of the immune system. Both receptors signal to activate the transcription factor nuclear factor kappa B (NF-κB) and

mitogen-activated protein kinase (MAPK) pathways inducing transcription of genes involved in a variety of cellular processes including inflammation, apoptosis and proliferation. Moreover, TNFR1 also contain a cell death domain signaling to induce apoptosis through caspase activation [24]. Through its receptors, TNF controls and orchestrates the immune response at several levels. TNF signaling is central in the pathogenesis of inflammatory and autoimmune diseases including RA and Crohns disease, and in cancer-associated chronic inflammation [24]. Overall, biologics targeting TNF signaling are successfully used in the treatment of chronic inflammatory disorders. However, adverse effects associated with global immune suppression give rise to concern [25].

1.1.3. Toll-like receptors

The initial recognition of infection by the innate immune system is best characterized in response to microbial infection sensed by pattern recognition receptors (PPRs) including TLRs [26]. TLRs are highly evolutionarily conserved with high homology to the Toll-gene found in *Drosophila melanogaster* [27] and are expressed by a variety of cell types [28]. The first evidence of human TLR involvement in pathogen recognition was reported in 1997 [29]. So far, fourteen mammalian subtypes are identified of which TLRs 1-10 are found in humans. TLRs are exogenously located on the cell surface (TLR1, TLR2, TLR 4, TLR6, TLR10) or endogenously located in endocytotic compartments (TLR3, TLR7, TLR8 and TLR9). TLRs detect conserved microbial motifs known as pathogen associated molecular patterns (PAMPs). The different TLRs recognize and respond to structurally different PAMPs, and the subcellular localization correlates to some extent to the type of ligands recognized by the specific TLRs (Figure 3). TLR2 dimerizes with TLR1 or TLR6 to discriminate between triacetylated and diacetylated bacterial lipoproteins, respectively. TLR6 recognizes lipipolysaccaride (LPS), whereas TLR5 recognizes flagellin. In contrast, the endogenous TLR3, TLR7, TLR8 and TLR9 are activated by nucleic acids-based PAMPs (dsRNA, ssRNA, CpG DNA) [28]. TLRs can also be activated by endogenous host-derived molecules in the absence of a microbial infection to induce "sterile" inflammation. These "self" molecules are referred to as danger associated molecular patterns (DAMPs) and may be

intracellular molecules released by necrotic cells or in response to tissue injury such as various proteins, fatty acids and nucleic acids. TLR2 recognizes a particularly diverse group of PAMPs and DAMPs due to its dimerization with TLR1 or TLR6 [28, 30].

Although recognized as a central part of the innate immune system, TLRs are also important regulators of adaptive immune responses [31]. Their involvement is linked to a variety of pathological conditions including inflammatory and auto-immune diseases such as systemic lupus, type I diabetes, inflammatory bowel disease, multiple sclerosis and RA [30, 32]. Moreover, TLRs are thought to contribute to the pathogenesis of diseases strongly associated with inflammation such as cancer and atherosclerosis [33, 34].

1.1.4. TLR signaling pathways

All TLRs, along with members of the IL-1 receptor family, contain a cytosolic Toll/IL-1 receptor (TIR) domain [18]. The differential signal transduction activated upon TLR ligand recognition depends on recruitment of different adaptor molecules including myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL, also called TIRAP), TIR-domain containing adaptor inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) [35]. With the exception of TLR3, MyD88 is recruited by all TLRs (and members of the IL-1R family) and is essential for TLR signaling. MyD88 further recruits the IL-1 receptor-associated kinases (IRAKs) IRAK1, IRAK2 and IRAK4 which leads to activation of ubiquitin ligase TNF receptor associated factor family 6 (TRAF6). TRAF in turn activates transforming growth factor β-activated kinase 1 (TAK1) to phosphorylate and activate the IκB kinase (IKK) complex. IKKβ phosphorylates IκB, the natural inhibitor of NF-κB. IκB phosphorylation leads to its ubiquitination and proteosomal degradation, allowing translocation of NF-κB to the nucleus to initiate inflammatory gene transcription. TAK1 can also activate members of the MAPK family including p38, ERK and JNK which in turn activate several transcription factors including AP-1 and CREB.
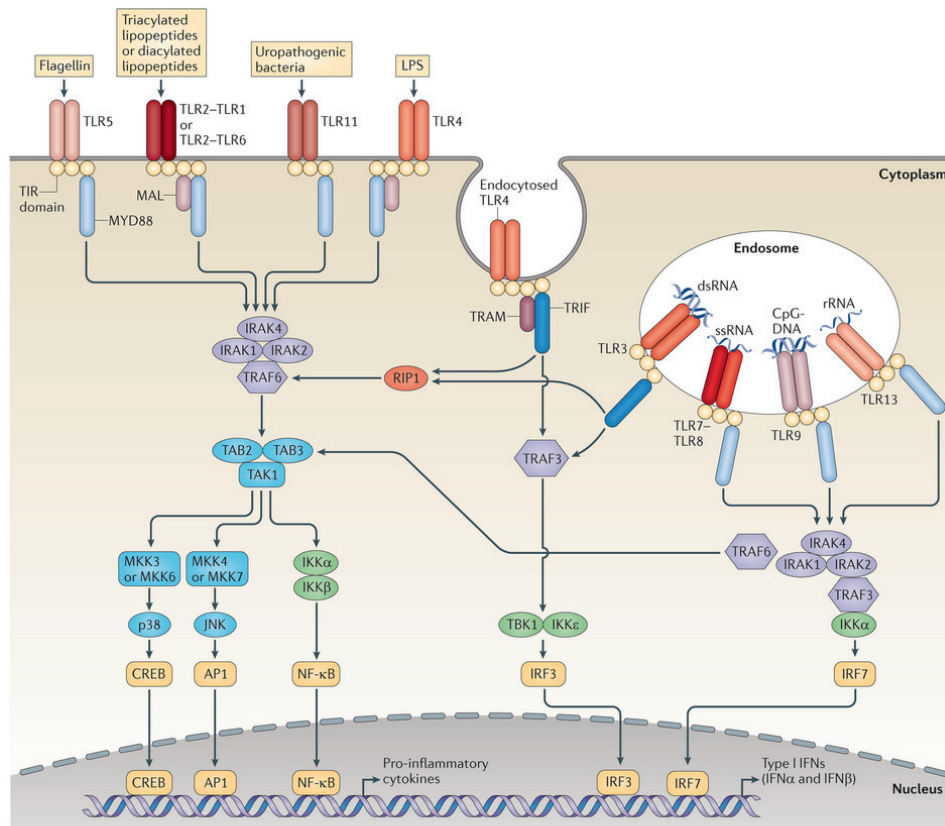
Figure 3: Schematic view of mammalian TLR signaling. TLR4, TLR5, TLR11 and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes, recognizing microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes. Upon ligand recognition, TLR signaling is effectuated by dimerization of receptors. Engagement of the distinct signaling adaptor molecules stimulates downstream signaling pathways leading to the activation of the mitogen-activated protein kinases (MAPKs), and to activation of transcription factors nuclear factor-κB (NF-κB), interferon-regulatory factors (IRFs), cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1). A major consequence of TLR signaling is the induction of pro-inflammatory cytokines, and in the case of the endosomal TLRs, the induction of type I interferon (IFN). dsRNA, double-stranded RNA; IKK, inhibitor of NF-κB kinase; LPS, lipopolysaccharide; MKK, MAP kinase kinase; RIP1, receptor-interacting protein 1; rRNA, ribosomal RNA; ssRNA, single-stranded RNA; TAB, TAK1-binding protein; TAK, TGFβ-activated kinase; TBK1, TANK-binding kinase 1. From O'Neill *et al.* 2013, with permission [36].

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These pathways can synergize in TLR-induced inflammatory gene expression by coordinated binding of transcription factors to AP-1 and NF-κB to binding sites found in the promoter sequences of several central inflammatory genes including IL-1β, IL-6 and TNF. The MyD88-independent pathway through which TLR3 and also TLR4 mediates their signaling, TRIF is recruited to induce activation of IRF3 and the production of type I interferons (IFNs) which are crucial in anti-viral responses [35]. A schematic overview of TLR signaling is presented in Figure 3.

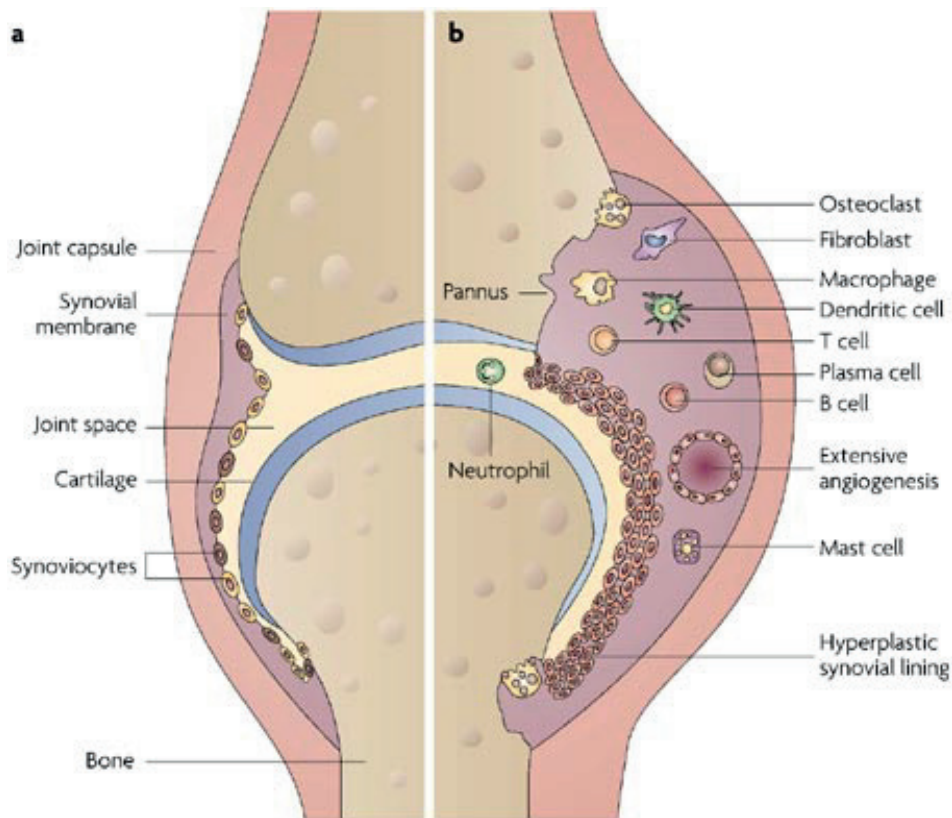### 1.1.5. Chronic inflammation and autoimmunity

The purpose of the inflammatory response is to eliminate invading pathogens, repair damaged tissue and to restore homeostasis. Dysregulation of the inflammatory processes or loss of tolerance against microbes and other environmental or endogenous factors that do not pose a threat, may shift the acute inflammatory response towards becoming chronic and pathological evoking disease and tissue damage rather than repair and resolution. Chronic inflammation can occur when the resolution process is disordered. Continuous infiltration and retention of leukocytes and excessive production of inflammatory mediators lead to persistent inflammation and eventually tissue destruction [37, 38]. There is a strong link between chronic inflammation and tumorigenesis; the longer inflammation persists, the higher the risk of developing certain types of cancer [39]. Why some inflammation resolve and some do not is a question not yet fully answered. Our understanding of the complex signaling regulation leading either to perpetuation and escalation or to resolution and termination of the inflammatory response is expanding, but still limited. Fibroblasts are the most common cells in connective tissue in animals and are proposed to play a particularly important role in regulating the transition from an acute resolving to chronic persistent inflammation by regulating leukocyte behavior and function [37].

Autoimmune diseases arise when the adaptive immune system fails to distinguish between "self" and "non-self" and is subsequently triggered by effectors (autoantibodies) produced by the host itself [40]. More than 70 different disorders including RA, psoriasis, systemic lupus erythematosus and multiple sclerosis are

categorized as autoimmune diseases, affecting approximately 5% of the Western world population. The immune system normally prevents self-antigens from stimulating an inflammatory response through a tightly regulated B-cell maturation process. The development of autoimmunity can occur as a consequence of a failure in B-cell tolerance maturation towards autoantigens, and leads to an increased number of circulating autoreactive B-cells [41].

1.1.6. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease affecting about 1% of the world's population. RA is 2-3 times more prevalent in women than in men, and cause disability, chronic ill-health and premature mortality. The aetiology of RA, like many other autoimmune diseases, is still only partially understood, but a variable involvement of genetic, epigenetic and environmental factors (such as potential but still unproven infectious agents) are implicated [42, 43]. RA is characterized by chronic inflammation of the synovium – synovitis – leading to pain, swelling and ultimately joint destruction if untreated. Autoantibodies such as rheumatoid factor, antikeratin antibodies and anticirruline antibodies are detected in RA [44]. Fibroblast-like synoviocytes (FLS) appear to be key players of the RA pathogenesis [45]. They are prominent drivers of inflammation through production of immunomodulating cytokines, chemokines and eicosanoids perpetuating inflammation and further influx of immune cells. Their invasive phenotype may even allow transmigration of FLS spreading RA to unaffected joints [46]. FLS directly contribute to joint destruction through production of matrix-degrading enzymes such as matrix metalloproteinases (MMPs) degrading articular cartilage [47]. They also express receptor activator of nuclear factor B ligand (RANKL) and osteoclast differention factor. FLS may thus not only activate osteoclasts, but also induce osteclastogenesis and subsequent bone destruction in RA [48-50]. A schematic overview over the healthy versus the rheumatic joint is presented in Figure 4.

Joint capsule

Synovial membrane

Joint space

Cartilage

Synoviocytes

Bone

a

b

Pannus

Neutrophil

Osteoclast
Fibroblast
Macrophage
Dendritic cell
T cell
Plasma cell
B cell
Extensive angiogenesis
Mast cell
Hyperplastic synovial lining

Nature Reviews | Drug Discovery

Figure 4: The normal versus the rheumatic joint. In healthy synovial joints[a], the synovial membrane lining the joint cavity is a delicate structure, only 2-3 cells deep. The synovial membrane is attached to skeletal tissues at the bone-cartilage interface, not connected to the surface of articular cartilage. Small quantities of synovial fluid (2.5 mL in a normal knee) coat the synovial surface and provide nutrition and lubrication to articulate free movement. In the rheumatic joint[b], immune cells such as CD^{4+} T-helper cells, B cells, neutrophils and macrophages infiltrate the synovium, producing cytokines activating the cells of the synovial lining, the fibroblast-like synoviocytes (FLS). The synovial membrane becomes hyperplasic, ultimately forming an invasive and destructive structure, pannus, which migrates onto and into the articular cartilage and underlying bone. A continuous production of pro-inflammatory cytokines and chemokines along with matrix-degrading enzymes and mediators of increased osteoclast activity and osteclastogenesis eventually leads to destruction of cartilage and bone. From Smolen *et al.* 2003, with permission [51].

During the last decade, increasing lines of evidence point to involvement of TLR signaling in RA pathogenesis [30]. In synovium from RA patients, levels of several TLRs are elevated compared to osteoarthritic patients or healthy controls [32, 52]. RA synovial cell cultures deficient in MyD88 and TIRAP adaptor molecules display significantly lower spontaneous production of proinflammatory cytokines such as TNF and IL6 along with several MMPs [53], indicating a role for TLR signaling in synovitis. Several DAMPs including heat shock proteins [54-56], high mobility group box 1 protein [57] and necrotic cells [58, 59] are detected in RA joints [60] and may thus contribute to TLR-induced sterile inflammation [61]. Moreover, TLR ligands of microbial origin, in particular bacterial peptidoglycans, are detected in RA joints [62, 63]. In animal models, injections of TLR ligands such as peptidoglycan, CpG DNA and dsRNA alone induce joint inflammation [64-66]. Accordingly, TLRs may contribute to RA pathogenesis by recognizing both microbial and host-derived ligands found in arthritic joints.

Revelation of the central roles of cytokines, TNF in particular, in RA pathogenesis led to the development of biological drugs targeting cytokine signaling networks. During the last decade, anti-TNF therapeutics such as Etanercept and Infliximab have been used for RA treatment with great success [25]. However, several drawbacks of these therapies have emerged. The degree of efficacy is inadequate as approximately 1/3 of patients do not respond well to treatment for unknown reasons. Adverse effects are associated with a global suppression of the immune system, such as opportunistic infections, and many patients suffer disease recurrence [25]. Accordingly, the search for novel therapeutic strategies is of great interest.

1.2. Bioactive lipids in inflammation

Lipids are important in our body and diet serving as source of energy, protecting our inner organs from injury and help us maintain stable body temperature. Moreover, cell membranes surrounding every single cell in our body are composed of fatty acids arranged in phospholipid bilayers. However, lipids are not only passive building blocks and energy reservoirs. Cells express hundreds of different enzymes active in the

biosynthesis of thousands of different lipids. The magnitude of lipid diversity is approaching that of proteins (10 000s); why cells invest energy into synthesizing such a complex lipidome is largely unclear, and we are only beginning to understand this diversity [67]. Many lipids are potent signal molecules regulating various biological processes including cell proliferation, apoptosis, metabolism and migration. Furthermore, bioactive lipids play pivotal roles in orchestrating initiation, perpetuation and resolution of the inflammatory response. The Ω6 fatty acid arachidonic acid (AA) is of particular importance as it is precursor for many bioactive lipid messengers regulating vital cellular processes including proliferation, apoptosis and inflammation (Figure 5) [68].

Structurally and historically, bioactive lipids can be sorted into three major classes [69]. Class I includes eicosanoids derived from AA; prostaglandins, leukotrienes, thromboxanes and their relatives [70]. These lipid mediators regulate a wide variety of physiological and pathological processes, and are traditionally considered mainly pro-inflammatory. Class II represents lipid mediators with either glycerol or sphingosine backbone including lysophospholipids and their derivatives (including platelet activating factor (PAF) and sphingosine-1-phosphate [71, 72]), and endocannabinoids [73]. Class III consists of anti-inflammatory lipid mediators derived from Ω3 fatty acids, including resolvins and protectins derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively [74]. Class I, II and III lipid mediators generally act though recognition by their specific G-protein coupled receptors (GPCRs) to elicit their biological activity [75-77]. In addition, specific GPCRs are identified that recognize medium- to long-chained free fatty acids [78], these may hence be regarded as another class of lipid mediators.

In 1893, the production of acetylsalicylic acid (launched as Aspirin) changed the course of anti-inflammatory therapy [79]. A new class of drugs, nonsteroidal anti-inflammatory drugs (NSAIDs), evolved and is still one of the most widely used therapeutic agents today. The mechanism of action however, was not revealed until 1971 when Sir John Vane reported the inhibitory effect of NSAIDs on the COX enzyme (at that time assigned the name COX1), subsequently reducing prostaglandin production [80]. COX enzymes are rate-limiting enzymes in the metabolism of AA to prostanoids,

including prostaglandins, prostacyclins and thromboxanes (Figure 5). Three COX enzymes are now known, COX1, COX2 and COX3 [81]. COX1 is considered to be more or less constitutively expressed and is found in nearly all tissues, while COX2 is induced by various pro-inflammatory stimuli [75]. The expression of COX2 is elevated in RA compared to normal and osteoarthritic synovium [82-84]. The COX3 enzyme is the most recently discovered and least studied of the three and its biological impact is still not clear. However, it has been suggested to produce anti-inflammatory members of the prostanoid family [85].

Imbalances in regulating lipid signaling pathways including eicosanoids, lysophospholipids and lysophospholipid-related mediators such as PAF is associated with a wide range of pathological conditions including allergy, chronic inflammation, autoimmunity, cancer, hypertension, metabolic and cardiovascular diseases, among others [68].

1.2.1. Phospholipase A2 (PLA2) enzymes

PLA2 enzymes catalyze the hydrolysis of membrane phospholipids at the *sn*-2 position releasing a fatty acid and a lysophospholipid, which are further enzymatically metabolized to various lipid mediators (Figure 5). The PLA2 enzyme superfamily have been known and studied for more than a century, and is extensively reviewed [86-88]. They were first discovered and described in cobra and rattle-snake venom at the end of the 19th century, and later in human pancreatic extracts. To date, more than 30 enzymes possessing PLA2 activity are identified in mammals. They are generally divided into six types due to biochemical properties; secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), Ca^{2+}-independent PLA2 (iPLA2), PAF acetyl hydrolase (PAF-AH), lipoprotein-associated PLA2 and adipose PLA2. Their diversity implicates PLA2 enzymes in a broad range of biochemical processes and pathological conditions, in large part associated with their activity releasing AA as precursor of Class I bioactive lipids [89].

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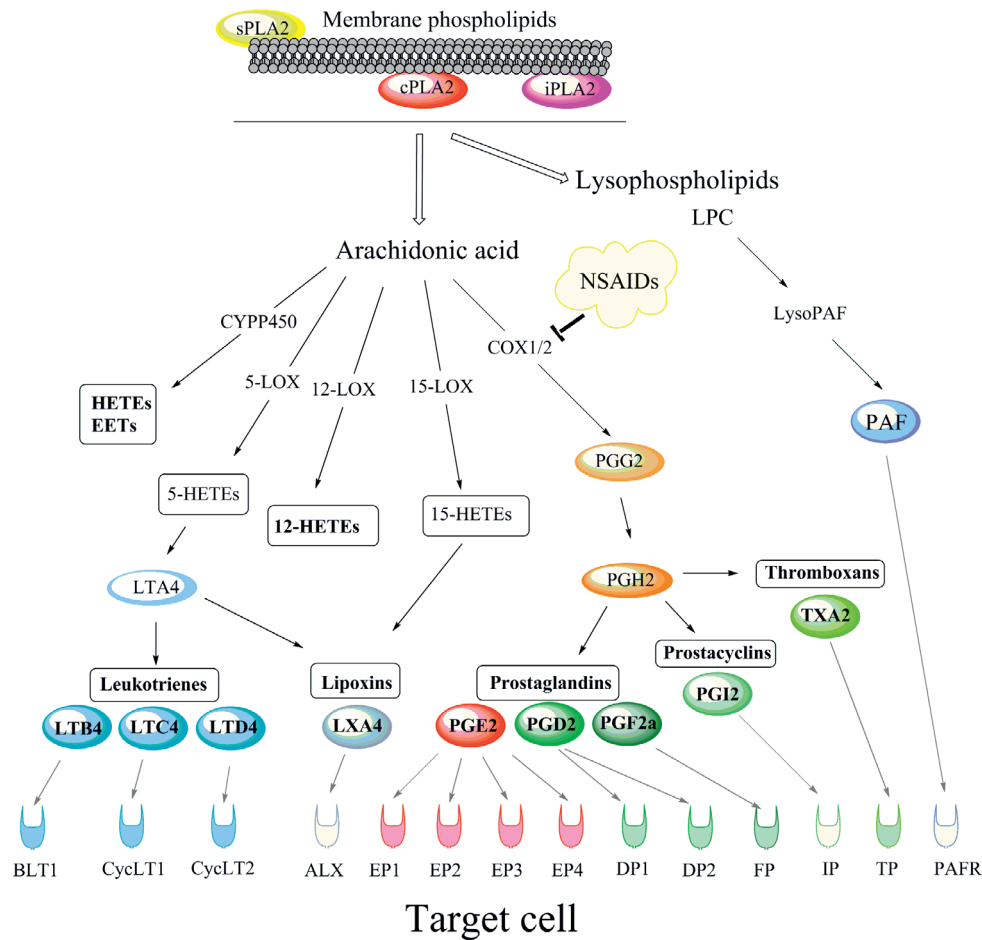
Figure 5: Lipid mediator pathways: PLA2 enzymes hydrolyze membrane phospholipids to release arachidonic acid (AA) and a lysophospholipid such as lyso-phosphatidylcholine (LPC). LPC can act as chemoattractant for immune cells, and can be enzymatically synthesized to form platelet activating factor (PAF). PAF is involved in angiogenesis, cell proliferation, osteoclastic bone resorption and inflammation. AA is metabolized through cytochrome P-450 (CYPP450), lipoxygenase (LOX) pathways or cyclooxygenase (COX) pathways to form bioactive eicosanoids including leukotrienes (LTs), lipoxins (LXs) and prostanoids including prostaglandins (PGs), prostacylins (PGIs) and thromboxans (TXs). Prostanoids are generally pro-inflammatory increasing vascular permeability and immune cell chemotaxisis, angiogenesis, adhesion and cell growth, whereas lipoxins exert anti-inflammatory effects. TXs facilitate platelet aggregation, while PGI2 prevents platelet aggregation. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX enzymes, blocking the production of pro-inflammatory eicosanoids. Lipid mediators act through their specific G-protein coupled receptors (GPCRs) on target call membranes. Modified from *Harizi et al.* 2008 [70] and *Murakami* 2011 (60) with permission.

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1.2.1.1. Cytosolic phospholipase A2

cPLA2s (group IV cPLA2) are large, cytosolic proteins (61-114 kDa) typically containing a C2 domain for Ca^{2+}-dependent association to the membrane [90]. The first identified cytosolic PLA2, now attributed to group IVA PLA2 (cPLA2α), was reported by Christina Leslie and Ruth Kramer in 1986 in neutrophils [91] and platelets [92], respectively. The cPLA2α enzyme and later purified, sequenced and cloned by James Clark and Ruth Kramer in 1991 [93, 94]. Since then, six cPLA2 subtypes are identified [86], but cPLA2α is still the most extensively studied isoform. cPLA2α is regulated by an increase in intracellular Ca^{2+} levels and by phosphorylation in response to various cellular stimuli [95]. The cPLA2α enzyme contains several important phosphorylation sites, including Ser505, Ser727 and Ser515. These sites are phosphorylated by mitogen-activated protein kinases (MAPKs), MAPK-interacting kinase (MKK1), and calcium-calmodulin kinase II, respectively [90]. In general, Ca^{2+}-binding induces translocation of cPLA2α to intracellular membranes including Golgi, ER and the nuclear envelope, while cPLA2α phosphorylation plays an important role in regulating catalytic activity releasing AA from membrane phospholipids [90].

Unlike other PLA2 enzymes, cPLA2α possesses acyl chain specificity for AA *in vitro* [93, 96, 97]. Due to its central role in AA release and subsequent eicosanoid production, cPLA2α activation is considered important in regulating normal and pathological processes in various tissues and cell types [98-100]. Studies in cPLA2α deficient mouse models and human cell cultures have indicated a role for cPLA2α in inflammatory diseases including collagen-induced arthritis [101, 102], asthma [103], pulmonary fibrosis [104], Parkinson [105] and other neurodegenerative diseases [106], and also in tumorigenesis [107, 108]. Accordingly, cPLA2α is proposed to be a potential therapeutic target in the treatment of inflammatory disease; however, the molecular mechanisms involved are still not elucidated. More research is needed to survey the processes in which cPLA2α activity is implicated, and downstream cellular effects of its inhibition.

1.2.1.2. Calcium-independent phospholipase A2

The Ca^{2+}-independent PLA2s (iPLA2s) have structural similarities with cPLA2, and these two types of enzymes are believed to have evolved from a common ancestral gene [87]. The iPLA2 enzyme was first characterized in macrophages in 1994 [109]. Since then, six human iPLA2 members are identified, diverse in structure and function [86]. Although GII, GIVC and GVIII all are display Ca^{2+}-independent activity, the term "iPLA2" only apply to GVI PLA2. iPLA2s activity is associated with processes including proliferation, apoptosis, bone formation and monocyte recruitment. iPLA2s are further believed to function as "house-keeper" enzymes maintaining lipid and membrane homeostasis within cells [86, 110]. iPLA2s lack substrate specificity, and can thus release a variety of fatty acids in addition to AA [111], including DHA, a precursor of resolvins involved in resolution of inflammation [112]. The iPLA2 enzymes are also associated with several pathological conditions, including diabetes, Barth syndrome and neurodegenerative disorders [86].

1.2.1.3. Secretory phospholipase A2

sPLA2s are low-molecular mass, Ca^{2+} requiring enzymes initially described in snake and bee venoms [113]. The purification and cloning of the first human, non-pancreatic secreted PLA2 in 1988 (designated type IIA secretory PLA2), more resembling the PLA2s known from rattlesnake venom than the known human pancreatic PLA2s, caught the eye of a broader scientific community [114, 115]. The human genome contains nine sPLA2 genes encoding catalytically active enzymes comprising PLA2 groups I, II, V, X and XII [86]. sPLA2s exhibit a large variety of cellular functions and their expression pattern vary greatly with cell type and tissue location. Central functions of sPLA2s are their role in host defense against bacterial infection due to their ability to kill gram-positive and Gram-negative bacteria [116], and their antiviral activity [117]. The sPLA2s also appear to be implicated in various inflammatory diseases [86]. Several sPLA2 isoforms are expressed and released by human immune cells including macrophages, monocytes, neutrophils, mast cells and T-cells. Elevated levels of various isoforms are detected at sites of inflammation, and in biological fluids and tissues from

persons with inflammatory or auto-immune diseases such as acute pancreatis [118], septic shock [119], inflammatory bowel disease [120] and RA [114, 121, 122].

Together with cPLA2α, sPLA2s are considered the primary PLA2s catalyzing AA release [123, 124] although iPLA2s can also contribute [125]. However, the catalytic effects of sPLA2 are not limited to AA release but may also include other unsaturated fatty acids such as oleic acid, linoleic acid and $\Omega3$ fatty acids (e.g. DHA and EPA, precursors of anti-inflammatory lipid mediators) [87]. The involvement of sPLA2 in the inflammatory response was initially believed to be in large part due to hydrolysis of AA as a substrate for the biosynthesis of proinflammatory lipid mediators. However, during the last decades alternative mechanisms for sPLA2s to activate immune cells and to induce production of inflammatory mediators have been identified [126]. These mechanisms are believed to be unrelated to the catalytic activity of sPLA2 and rather due to interaction to specific or promiscuous receptors or surfactant protein on cell membranes [127].

1.2.2. Platelet-activating factor

PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) was first described in 1972 to induce platelet aggregation and histamine production in rabbit basophils [128]. PAF can be synthesized via *de novo* or remodeling pathways; *de novo* synthesis provides small physiological amounts of PAF for normal cell function, whereas the remodeling pathway is believed to be the primary route of PAF synthesis in response to pro-inflammatory stimuli and stresses such as UV-radiation [129, 130]. PAF is not stored, but is rapidly synthesized in response to stress. cPLA2α is a key enzyme in the remodeling pathway of PAF synthesis as demonstrated in independent knock-out animal models [103, 131]. cPLA2α activity removes the *sn-2* residue of phosphatidylcholine, leaving a molecule of lyso-PAF which is further metabolized to biologically active PAF (Figure 5) [132]. Moreover, PAF is in turn a potent inducer of PLA2 activity and subsequent AA release, creating a positive feed-back loop [133-135].

PAF signals through the PAF receptor (PAFR) which is expressed on the cell surface of a wide variety of cells [135]. Signaling mediated downstream the PAFR include activation of phospholipases PLA2, PLC and PLD, protein kinase C, phosphatidylinositol 3-kinase, protein tyrosin kinases, MAPKs and the second messengers inositol triphosphate, diacylglycerol, calcium and cyclic adenosine monophosphate (cAMP) [135]. In addition, PAFR independent signaling can also occur [136]. Through its downstream mediators, PAF can activate transcription factors including AP-1 and NF-κB, leading to transcription of inflammatory genes [137-139]. PAF is considered a potent mediator of the inflammatory process [140], and elevated levels of PAF is reported in inflammatory diseases such as Crohns disease [141], psoriasis [142], ischemic injury [143] and asthma [144]. However, its name is today somewhat misleading since PAF elicits a variety of physiological and pathological actions besides acting as a pro-inflammatory lipid messenger [145]. PAF is associated with apoptosis [146], cell proliferation [147], reproduction [148], osteoclastic bone resorption [149], tumor growth, metastasis and angiogenesis [150, 151], and beneficial effects of blocking the PAFR is shown in several types of cancer including breast cancer and melanoma [152]. PAF is also proposed to be involved in keratinocyte proliferation and wound healing [153, 154].

PAF is deactivated by a group of PLA2 enzymes, PAF-AH, and the degradation of PAF by PAF-AH is demonstrated to inhibit severe acute inflammation [155]. A decreased plasma level of PAF-AH is associated with several diseases including asthma [156], systemic lupus erythematosus [157], juvenile RA [158] and Crohn's disease [159]. These observations indicate a harmful effect of an increase in pathological levels of PAF as a consequence of reduced PAF-AH activity. In contrast, detection of elevated PAF-AH levels are reported in pathological conditions associated with inflammation including RA [160] and diabetes [161]. Moreover, exogenous (recombinant) PAF-AH is recently shown to play a central role in reducing inflammatory injury and clearance of bacteria in a murine model of sepsis [162]. This may represent a defensive strategy to compensate for elevated levels of PAF induced by the inflammatory process [163]. Indeed, PAF-AH is secreted by hematopoietic cells in response to pro-inflammatory stimuli [164], and PAF-AH display neuroprotective properties in mouse ischemia [165].

1.2.3. Prostaglandin E2

PGE2, synthesized from AA by the COX enzymatic pathway, is the most abundant prostaglandin and has been recognized as a biologically active immunoregulator since the 1960s [166]. Its biological effects are ubiquitous in both physiological and pathophysiological mechanisms including nerve growth, nerve and brain functions, wound healing, bone metabolism, fever, pain and immune responses, and is associated with diseases including Alzheimer's disease, RA and cancer [69, 167]. The central role of PGE2 in inflammation is emphasized by the effective symptomatic relief by NSAIDs in patients suffering from chronic inflammation such as RA [168], which is in large part attributed to reduced PGE2 synthesis [69]. PGE2 is a known immunoactivator [169], and promote immune cell influx to inflamed tissues and inflammatory angiogenesis [170, 171]. In the joint, PGE2 is shown to mediate chronic joint inflammation in collagen-induced arthritis (CIA) in mice [172] and CIA mice deficient in mPGES-1, the enzyme converting PGH2 into PGE2 (Figure 5), display reduced disease activity [173]. PGE2 is also shown to induce inflammatory gene expression in RA synovial fibroblasts [174]. PGE2 is furthermore proposed to be a critical factor in bone and cartilage resorption in arthritis through regulation of osteoclast activity and expression of cytokines and MMPs in various model systems [175-177]. However, the actions of PGE2 are highly pleiotropic. PGE2 can also exert anti-inflammatory and immunosuppressive effects, possibly dependent on the cell type and receptor subtypes involved [167, 178-180]. Furthermore, PGE2 can act in concert with other prostanoids to modulate inflammation in a context-dependent manner to shift the whole process in both anti-inflammatory and proinflammatory directions [181].

PGE2 signals through four substrate-specific GPCRs; EP1, EP2, EP3 and EP4 [181]. EP2 and EP4 increase intracellular cyclic AMP (cAMP) levels inducing protein-kinase A activation, while EP3 decreases cAMP levels. The effects of PGE2 are believed to be cell- and tissue specific due to this receptor diversity, receptor distribution and pathway cross-talk. EP3 exists in several splicing variants, and may thus constitute a key to the pleiotrophic effects attributed to PGE2 [182]. EP receptors can also transactivate the epidermal growth factor receptor (EGFR) and downstream signaling cascade [183, 184]. In addition, PGE2 is recognized by nuclear receptors

including peroxisome proliferator activated receptors (PPARs) [185]. This diversity in receptors, variability in their expression and pathway cross-talk may explain why one mediator like PGE2 can elicit such pleiotropic responses.

2. Study objectives

Chronic inflammatory diseases represent a major challenge and are a common cause of decreased life quality and premature mortality. However, the aetiology of many autoimmune and chronic inflammatory diseases (including RA and psoriasis) is still largely unknown, and the therapeutics available are associated with adverse effects and unsatisfactory efficacy. The inflammatory response involves many different cell types, and a finely tuned production of pro-inflammatory, anti-inflammatory and resolving mediators. The shift from a beneficial well-regulated inflammation resulting in resolution and tissue repair towards a pathological chronic inflammation can be triggered by small imbalances in this highly complex signaling network. Bioactive lipids and enzymes catalyzing their biosynthesis are becoming increasingly interesting as our understanding of their roles orchestrating inflammation and immune responses expands. In order to understand the pathology of chronic inflammatory diseases and to develop novel therapeutic strategies, it is of crucial importance to understand the molecular signaling mechanisms and relationships between cells, processes and signaling pathways involved.

The overall objective of this work was to investigate the role of cPLA2α and lipid messengers in molecular mechanisms involved in chronic inflammatory disease. As this thesis emerged from the substantial research performed by Berit Johansen and the PLA2 research group concerning involvement of PLA2 enzymes in cellular signaling mechanisms typical for psoriasis, the role of PAF was investigated in a keratinocyte model system (Paper I). We next established a cellular model system for RA, directing our focus towards investigating the role of PLA2 enzymes, cPLA2α in particular, in central signaling pathways relevant for chronic joint inflammation (Papers II-IV).

The aims of this thesis were as follows:

Aim 1: To elucidate the cellular consequences of PAF stimulation – what is the predominant role of PAF in differentiated keratinocytes? (Paper I).

Aim 2: To investigate the involvement of cPLA2α enzyme activity in TNF-induced signaling in synoviocytes (Paper II).

Aim 3: To investigate the involvement of cPLA2α and possibly other PLA2 subtypes in TLR2-induced signaling (Paper III).

Aim 4: To investigate involvement of MAPKs in TLR2-induced cPLA2α activation, and whether NF-κB is implicated in TLR-induced, cPLA2α-dependent cytokine production.

Aim 5: Can COX-derived prostanoids, such as PGE2, act as messengers downstream cPLA2α activity in regulating TLR2 signaling? (Paper IV).

3. Summary of papers

Paper I

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid-derived signaling molecule with highly pleiotrophic effects. PAF is believed to be implicated in several pathological conditions including psoriasis, inflammation and allergy, and may also participate in mitogenic events and cancer development. Increased levels of PAF are found in psoriatic skin, but PAF's exact role in epidermis is uncertain. In paper I, we aimed to investigate the physiological consequences of excess PAF production in epidermis. We examined the gene regulatory effects of PAF short-term stimulation in differentiated HaCaT keratinocytes by transcriptional profiling. Even though PAF induces COX2 expression, we found that PAF regulates only few genes associated with inflammation in differentiated keratinocytes. Rather, we show that natural PAF rapidly regulates genes involved in proliferation, (anti)-apoptosis and migration, all sub-processes of re-epithelialization and wound healing. Moreover, profiling of phosphorylated kinases, cellular wound-scratch experiments, resazurin assay and flow cytometry cell cycle phase analysis all support a role for PAF in keratinocyte proliferation and epidermal re-epithelialization. In conclusion, these results suggest that PAF acts as an activator of proliferation and may, therefore, function as a connector between inflammation and proliferation in differentiated keratinocytes.

Paper II

Rheumatoid arthritis (RA) is an inflammatory joint disease characterized by chronic synovitis causing pain, swelling and loss of function due to destruction of cartilage and bone. The complex series of pathological events occurring in RA are largely regulated via excessive production of pro-inflammatory cytokines, the most prominent being tumor necrosis factor (TNF). The objective of this work was to elucidate the involvement of group IVA cytosolic phospholipase A2 (cPLA2α) in TNF-induced regulation of synovitis and joint destructive effectors in synoviocytes (synovial

fibroblast-like cells), as a cellular model system for RA synovium. Inhibitors of cPLA2α enzyme activity (AVX002, ATK) significantly reduced TNF-induced cellular release of AA, PGE2, IL8 and MMP3. This reduction was evident both at transcriptional, protein or metabolite levels. Interestingly, cPLA2α inhibition affected several key points of the arachidonyl cascade; AA-release, cyclooxygenase-2 (COX2) expression and PGE2 production. Furthermore, our results suggest that cPLA2α is subject to transcriptional auto-regulation as inhibition of cPLA2α resulted in reduced PLA2G4A gene expression in TNF-stimulated synoviocytes. Thus, cPLA2α appears to be an important regulator of central effectors of inflammation and joint destruction, namely MMP3, IL8, COX2, and PGE2. Decreased transcription of the PLA2G4A and COX2 genes in response to cPLA2α enzyme inhibition further suggest a self-reinforcing effect of cPLA2α inhibition in response to TNF. Collectively, these results suggest that cPLA2α is a modulator of synovitis as its inhibition reduces the production of multiple key pro-inflammatory factors involved in RA pathogenesis.

Paper III

In rheumatoid arthritis (RA), toll-like receptors (TLRs) contribute to synovitis by recognizing damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) found in RA joints. Studies in cell cultures, animal models and clinical trials point to TLR2 as a driver of synovial inflammation and joint destruction in RA. However, few studies have investigated the implication of PLA2 activity in TLR-induced signaling in RA synovium. Having shown the involvement of cPLA2α activity in TNF-induced synoviocyte inflammatory and joint-destructive mediators, we now aimed to characterize PLA2 enzyme involvement in TLR2-induced signaling. TLRs1-7 and a range of sPLA2, iPLA2 and cPLA2 enzymes were found to be transcriptionally expressed in cultured synoviocytes. Activation of TLR2/1 and TLR2/6 led to phosphorylation of cPLA2α at Ser^{505}, and induced AA release and PGE_2 production; effects that were attenuated by cPLA2α inhibitors. In contrast, sPLA2 inhibitors did not affect AA or PGE2 release. cPLA2α inhibitors furthermore attenuated TLR-induced expression of IL-6, IL-8 and COX2. Our results demonstrate for the first

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time that cPLA2α is involved in TLR2/1- and TLR2/6-induced AA release, PGE2 production and pro-inflammatory cytokine expression in synoviocytes. As a modulator of TLR2 signaling, our results expand the understanding of cPLA2α as a regulator of inflammatory molecular mechanisms in synoviocyte signaling.

Paper IV

In Paper III, we show that cPLA2α acts downstream the TLR2/6 ligand FSL-1 in regulating AA release, PGE2 production and IL-6 protein levels in human synoviocytes. Here, we aimed to investigate this signaling pathway in more detail. We found MAPKs p38 and ERK1/2 to be involved in FSL-1-induced cPLA2α phosphorylation at Ser^{505}, and in subsequent AA release and PGE2 production. Inhibition of cPLA2α further attenuated FSL-1-induced IκBα degradation, as did cyclooxygenase (COX) inhibition, indicating a role for both cPLA2α and COX-derived eicosanoids in regulating TLR2-induced NF-κB activation and hence subsequent transcription of NF-κB-regulated IL-6. Indeed, COX inhibition attenuated FSL-1-induced IL-6 transcription and protein production comparable to cPLA2α inhibition. Moreover, exogenously added PGE_2 alone induced IL-6 production and completely rescued IL-6 transcription when added simultaneously with FSL-1 in the presence of a cPLA2α inhibitor. Collectively, these results provide novel insights into the mechanism that cPLA2α, activated by p38 and ERK1/2, regulates TLR2-induced NF-κB activation and IL-6 production, possibly through COX/PGE2-dependent pathways.

4. General discussion

Diving into the world of lipid signaling is fascinating, challenging, at times confusing and provoke an intriguing admiration for the exquisitely fine-tuned regulation essential for our well-being. The inflammatory response and our immune system involve cross-talk between many signal-transduction pathways. Lipolytic enzymes (i. e. enzymes catalyzing the hydrolysis of lipids) and bioactive lipids have emerged as signaling "hot-spots" orchestrating many of these immunological processes and the shift between a normal, resolving inflammation towards a chronic pathological inflammation. However, the complexity of lipid signaling and cellular consequences of their actions is not fully elucidated. Lipid signaling is complex, redundant and often interconnected and even counter-regulated. In order to develop future therapeutic strategies and pin-point novel therapeutic targets in chronic inflammatory disease, we need to understand these underlying signaling mechanisms and pathway interactions.

A detailed discussion concerning the significance of our results in relevance to the specific model systems and inducers are discussed in detail in the respective papers. In this general discussion I will first briefly address the main findings in Paper I with focus on PAF-mediated effects in keratinocyte proliferation and wound healing (Aim 1). Next, Papers II-IV will be discussed in view of joint inflammation and RA, and the overall significance of our findings (Aims 2-5). The discussion will then be more generalized towards addressing the question of bioactive lipids as connectors between inflammatory and proliferative processes, in light of our findings in Papers I-IV. Finally, the future of cPLA2α as a potential therapeutic target will be discussed.

4.1. PAF - a promotor of proliferation and epidermal wound healing

Traditionally, PAF is considered a pro-inflammatory mediator, and is elevated in psoriatic scale [142] and dermatitis [186]. Expression of PAFR is also elevated in psoriatic lesions compared to normal skin [187], indicating a role for PAF in psoriasis pathogenesis. However, as described in Paper I we found few indications of a pro-inflammatory role of PAF in differentiated keratinocytes. Our results rather suggest that PAF primarily signal to induce transcription of genes associated with increased mitotic

activity, survival and migration, and that PAF promotes a re-entry of differentiated keratinocytes into a proliferative state; all mechanisms important in epidermal wound healing. The process of wound repair and tissue regeneration is characterized by inflammation including infiltration of immune cells, angiogenesis, re-epithelization and tissue remodeling [10]. Lipid mediators such as eicosanoids, endocannabinoids and sphingolipids play critical roles in regulating wound healing and tissue repair through modulation of the inflammatory process [11]. The role of PAF in regulating proliferative responses in keratinocytes is not unknown; in transgene keratinocytes expressing PAFR, accelerated proliferation was induced [147], and PAFR activation can induce proliferative effects through cross-talk with EGFR signaling [153]. However, there has been contradictory reports suggesting anti-proliferative effects of PAF/PAFR signaling [154]. As discussed in detail in Paper I, our results indicate that the proliferative effects of PAF are differentiation-dependent, thus providing a possible explanation for these contradictory findings. As PAF is produced in the initial inflammatory phase of wound healing, our results suggest that PAF/PAFR signaling links the inflammatory and proliferative processes, thus representing interesting therapeutic targets regarding impaired wound healing and pathological hyperproliferation such as dermatoses and cancer.

In RA, hyperproliferation of the synovial membrane is a hallmark. Little is known about the role of PAF in the joint, but levels of PAF are elevated in the synovial fluid of RA patients compared to osteoarthritic patients [188]. PAF is assigned a pro-inflammatory role in animal models of arthritis, as a PAFR antagonists display anti-inflammatory effects and reduced cartilage destruction [189, 190]. In osteoarthritic chondrocytes, PAF can activate both AA selective and non-selective PLA2 enzymes [191]. Unpublished data from our lab show that synoviocytes respond rapidly and strongly to PAF stimulation, both by increased AA release and by the transient morphology change described in Paper I. These responses in synoviocytes are induced by even lower PAF concentrations than in keratinocytes. There are no reports investigating a role of PAF in the in inflammatory synovial hyperproliferation; based on our findings, a possible proliferative effect of PAF in RA synovium is an interesting hypothesis to be addressed in future studies. Moreover, as PAF is both a product of, and

an activator of cPLA2α activity, it would be of interest to investigate the impact of cPLA2α inhibition on PAF production, and subsequent cellular consequences.

4.2. cPLA2α as a regulator of synovial inflammation and joint destruction

PLA2 enzymes along with other lipolytic enzymes metabolizing lipid mediators from AA or lysophospholipids have received increasing attention during the last decades and are highly relevant in the search for novel therapeutic approaches in treating inflammatory disorders. Lipid mediators generated downstream cPLA2α activation gives rise to a diversity of responses, many of which are pro-inflammatory and associated with various chronic inflammatory and autoimmune diseases as described introductory. Several groups have reported a pathologic role of cPLA2α in murine models of inflammatory arthritis [101, 102, 192], and ameliorating effect of chemical cPLA2α inhibition [193, 194]. However, the underlying mechanisms are yet to be elucidated. Synovitis and synovial hyperplasia are key features of RA and FLS derived from RA joints display an aggressive hyperproliferative phenotype, producing a variety of cytokines, chemokines and matrix-degrading enzymes driving synovitis and joint destruction [47]. Accordingly, FLS are considered key players in RA pathogenesis driving synovitis and joint destruction [45]. In Papers II-IV we applied the synovial fibroblast cell line SW982 (originating from a synovial sarcoma) as model system for RA synovium [195]. Fibroblasts in general are believed to play a key role in the development of chronic persistent inflammation [38, 196], and quiescent fibroblasts (as experimentally used in these studies) are shown to be particularly responsive to pro-inflammatory stimuli, hence providing a useful tool to study these signaling mechanisms [197].

In order to investigate the orchestrating role of cPLA2α in RA joint inflammation, we focused on two central pro-inflammatory stimuli; TNF and TLR2 activation. The central role of cytokines, particularly TNF, in inflammation is well documented, and TNF-blocking biologics are widely used in the treatment of RA along with other inflammatory diseases including psoriasis, psoriatic arthritis, ankylosing spondylitis, and Crohn's disease [25]. TLRs are important regulators of both innate and

adaptive immune responses, and dysregulation of their activation has emerged as an important factor in inflammatory and autoimmune diseases including, asthma, systemic lupus erythematosus (SLE) and RA [198]. Expression levels of several TLRs including TLR2 are elevated in RA [50, 52] and in particular at sites of cartilage destruction [199]. Moreover, several PAMPs and DAMPs known as TLR2 or TLR2-heterodimer ligands are found in RA joints [56, 60, 62]. Thus, the TLR2/1 and TLR 2/6 ligands Pam$_3$CSK$_4$ and FSL-1 were selected for the initial investigations presented in Paper III. Investigating the involvement of PLA2 enzymes and lipid messengers in TNF- and TLR2- induced signaling in synoviocytes as is done in Papers II-IV can provide valuable insights into important molecular mechanisms in RA pathogenesis. As we aimed to investigate the signaling downstream TLR2 activation in more detail in Paper IV, FSL-1 was selected due to its more potent induction of AA release.

cPLA2α is a key enzyme in synoviocyte AA release and subsequent PGE2 production in response to both TNF (Paper II) and TLR2 activation (Paper III). Our results further suggest that cPLA2α regulate TNF and TLR2-induced COX2 transcription; thus possibly affecting PGE2 synthesis both directly through providing AA as substrate for biosynthesis, and indirectly by regulating expression of the COX2 enzyme which is rate-limiting in PGE2 biosynthesis [75]. Transcriptional regulation of COX2 by cPLA2α has previously been shown in murine model systems [194, 200], supporting our data.

In Paper II we found that inhibition of cPLA2α attenuated its own transcription in response to TNF. The promoter region of the PLA2G4A gene contains binding sites for NF-κB, and cPLA2α is produced in response to IL-1β-induced NF-κB activation [201]. In Paper IV, we found cPLA2α to regulate NF-κB transcriptional activity, a mechanism also reported in other cell types [100, 202-204]. Our results may thus suggest that activated cPLA2α can stimulate its own transcriptional expression in a feedback-loop involving NF-κB in response to cytokines and possibly TLR2 activation. Together, our data propose that inhibition of cPLA2α activity may have a self-reinforcing effect on AA release and subsequent PGE2 production through negatively regulating both COX2 and cPLA2α expression.

In Papers III-IV we investigate the involvement of cPLA2α in TLR2-induced IL-6 expression. IL-6 is proposed to play a role in the shift from acute towards chronic inflammation [205] and is involved in synoviocyte activation, driving synovitis and joint destruction in RA [206, 207]. Increased levels of IL-6 are detected both in the joint and systemically in RA patients [208, 209] and experiments from various model systems support a pathological role for IL-6 in RA [210]. IL-6 deficiency provides protection against development of murine CIA [211, 212], and anti-mouse IL-6 monoclonal antibody suppress CIA development [213]. In humans, anti-IL-6 therapy improves symptoms of RA [198, 206, 214]. Our results demonstrating that synoviocyte IL-6 levels is partly controlled by cPLA2α in response to TLR2 activation, further support a role for cPLA2α as a central regulator of molecular mechanisms in synovial inflammation.

Our results in Papers II-IV clearly demonstrate that cPLA2α inhibition potently reduces PGE2 levels, which, considering the pleiotropic roles of PGE2 in RA and inflammation in general, indicate a potential anti-inflammatory effect. Our results further indicate that PGE2 may be an important actor downstream cPLA2α in regulating at least TLR2-induced IL-6 levels. This mechanism is to our knowledge not previously described in synoviocytes. Moreover, as NF-κB is central in inflammation in general and RA in particular [215, 216], our finding that cPLA2α can modulate synoviocyte NF-κB activation, possibly through COX/PGE2, is important expanding our understanding of the molecular mechanisms regulated downstream cPLA2α activity.

Degradation of cartilage and bone is a hallmark of RA. Of the metabolites we found regulated by cPLA2α activity (Papers II-IV), several are known to be involved in RA joint destruction. MMP3, with its wide range of substrate specificity and ability to activate other MMPs, is essential in RA cartilage degradation [217-219]. Osteoclasts are the main cells mediating focal bone erosion in RA. PGE2 is proposed to be a central factor in bone and cartilage resorption in arthritis through regulation of osteoclast activity and expression of cytokines and MMPs in various model systems [175-177]. IL-6 is implicated in osteoclast differentiation through its soluble receptor sIL-6R [220], possibly in cross-talk with PGE2 signaling as reported in murine osteoclast cell

cultures [221]. Our results thus suggest that cPLA2α regulates the expression of metabolites involved in RA joint destruction.

cPLA2α is proposed to be a regulator of neutrophil recruitment in murine CIA [192]. In Paper II, we show that cPLA2α is involved in TNF-induced production of the chemokine IL-8 which is overexpressed in RA synovium and acts as a chemoattractant for neutrophils [222, 223]. This finding suggests a possible mechanism through which cPLA2α may affect neutrophil recruitment in RA. Furthermore, IL-8 acts as an angiogenic factor. Angiogenesis, the formation of new blood vessels, is crucial in many physiological processes including reproduction, development and tissue repair [224]. In RA, along with many other pathologic condition including obesity, cancer and psoriasis, the angiogenetic process is dysregulated and excessive formation of new blood vessels is important in disease development [225]. Along with IL-8, COX2 and PGE2 are described as actors in the angiogeneic process [226]. Also IL-6 is shown to promote angiogenesis in RA [227]. Our results presented in Papers II and III demonstrate a regulatory role for cPLA2α in the production of all of these factors, thus indicating a role for cPLA2α in modulating synovial angiogenesis.

To sum up, our findings in Papers II-IV suggest that cPLA2α hold a key position in synoviocytes regulating pro-inflammatory, angiogenetic and joint destructive signaling downstream TNF and TLR2 activation, both important pathological signaling pathways involved in RA pathogenesis.

4.3. cPLA2α and bioactive lipids – links between chronic inflammation and cancer

The association between inflammation and cancer was first proposed in 1863 by Rudolf Virchow who noted that tumors tended to develop at sites of chronic inflammation [228]. Today, a substantial and growing amount of data reveals the importance of inflammatory processes in cancer initiation and progression [229]. Chronic inflammation predisposes to a variety of cancers, and 15-20% of all cancers worldwide are estimated to origin from underlying infections and inflammatory responses [228]. There are two pathways by which inflammation and cancer are viewed to be linked;

extrinsic pathways where smouldering inflammation increase cancer risk, and intrinsic pathways where dysregulated activity of oncogenes or tumor-suppressor genes causes inflammation and tumor development [230]. The body's response to tumors have many similarities with a healing wound; following tissue damage, cell proliferation is enhanced while the tissue regenerates. When the repair is complete or the assaulting agent is removed, inflammation and proliferation recede to resume tissue homeostasis. In tumors, like in impaired wound healing, this resolution fails and cells continue proliferating in an environment rich in inflammatory cells secreting inflammatory cytokines and growth/survival factors. In that sense, tumors resemble wounds that fail to heal [231].

In Paper I, we investigated the effects of PAF in keratinocytes, primarily revealing proliferative rather than pro-inflammatory responses, presumably directing cell signaling towards keratinocyte dedifferentiation, wound-healing and tissue repair. However, these findings may also suggest PAF as a link between inflammation, hyperproliferation and possibly even cancer development. PAF is indeed detected in elevated levels in colorectal carcinoma [150]. Furthermore, PAF regulates vascular endothelial cell migration and invasion [232], and is proposed to contribute to tumor growth in breast cancer [151] and prostate cancer [233] through enhanced cell motility and proliferation. PAF is also found to be a potent promotor of tumor angiogenesis [152] and is attributed a promoting role of skin melanoma metastasis [152, 234, 235]. In support of its role in skin cancer, PAFR transgenic mice display progressive hyperproliferative changes in the epidermis [147]. Inhibiting the PAFR furthermore efficiently inhibits proliferation of breast cancer cells [236]. Our results in Paper I revealing novel aspects of PAF-induced cellular responses in differentiated keratinocytes may thus complement the understanding of PAF as a regulator of hyperproliferation, cell migration and possibly tumorigenesis. However, psoriasis, although a chronic inflammatory disease, is rarely, if ever, accompanied by skin cancer [237]. This illustrates that our understanding of the inflammatory process and links to cancer development is still incomplete.

PAF is only one of many lipids generated downstream cPLA2α activation implicated in proliferative responses and tumorigenesis. Prostanoids play important

roles also in proliferation and tissue regeneration [11]. PGE2, PGF2α and TBX2, three main products of the COX enzymatic pathway, are proposed to be implicated in prostate cancer tumorigenesis through regulating proliferation, angiogenesis, metastasis and invasion [238-240]. PGE2 levels in particular are elevated in human tumors and is associated with regulation of leukocyte function, increased angiogenesis, metastasis, survival responses and cell cycle regulation [167, 241-243]. Accordingly, NSAID treatment is associated with decreased initiation and progression of tumorigenesis, and thus reduced mortality in several cancer types, including colorectal cancer [244-246]. Also in murine cancer models, COX enzymes appear to play crucial roles in tumor development and progression [247]. In view of the significance of COX enzymes and eicosanoids in development of many human cancers, it is not surprising that cPLA2α, a major provider of AA substrate for COX enzymes, is suggested to be implicated in tumorigenesis [108]. Several studies in murine models of cancer have pointed to a role for cPLA2α in tumor development and progression [107, 247-250]. The mechanisms by which cPLA2α impacts tumor growth are not fully understood, but regulation of tumor angiogenesis is proposed as a central factor [107, 251]. Having found cPLA2α to regulate multiple angiogenetic factor in synoviocytes, our results may contribute to the understanding of cPLA2α as an angiogenetic modulator.

Besides serving as a key enzyme in AA release and PGE2 production, we found cPLA2α to regulate expression of IL-6, IL-8, and MMP3. In addition to playing important roles in inflammatory and joint destructive processes, these factors are also implicated in tumorigenesis. IL-6 plays a central part in the pathogenesis of several types of cancers [22]. To mention some, differential TLR-dependent IL-6 production is proposed to count for reduced liver cancer risk in females, due to an inhibitory effect of estrogen [252], and IL-6-induced cell proliferation and anti-apoptosis signaling plays a pivotal role in early colorectal cancer tumorigenesis [253-255]. It is of interest that cancer involving aberrant IL-6 signaling (e.g. colon, liver, lung) are linked to TLR activation and inflammation [252, 256-259]. Due to its angiogenetic effects, IL-8 is associated with increased tumorigenesis [260], and is, through activating its receptor CCXR7, reported to phosphorylate EGFR to induce the mitogenic cascade promoting prostate tumor growth [261]. Overexpression of MMPs characterize most malignant

tumors, and they are directly involved in tumor invasion and metastasis [262]. Moreover, we found cPLA2α to modulate NF-κB activation. NF-κB is a crucial regulator of both carcinogenesis and inflammation, and provide a mechanistic link between these processes [263]. The observations of cPLA2α-dependent regulation of these factors may thus contribute to enlighten potential mechanisms through which cPLA2α can modulate tumorigenesis.

In Papers II-IV, cPLA2α involvement in TNF and TLR signaling pathways were in focus. While playing central roles in inflammation, both TNF and TLRs induce important signaling pathways and mediators implicated in tumorigenesis. The role of TNF in cancer development is janus-faced; its signaling can induce both positive and negative effects in cancer [264]. Despite its name generated from its initial discovery as being toxic to tumor cells, TNF also displays a potent tumor-promoting effect in mice [265]. TNF can promote cell survival, invasion, angiogenesis and tumor initiation [266, 267], and is produced in excessive amounts by malignant cells in experimental and human cancers during tumor growth and spread [265, 266, 268, 269]. Furthermore, clinical trials suggest TNF blockade to be a useful therapeutic strategy in cancer treatment [270, 271].

The traditional view of TLR signaling is the modulation of host defense and induction of inflammation in response to invading pathogens and tissue damage. However, it has become evident that the effects of TLR activation extend past inducing inflammation to include processes such as proliferation, survival and migration. TLR signaling may thus link inflammation to tissue repair, wound healing and cancer development [272]. TLR ligands augment proliferation, angiogenesis and metastasis and decrease apoptosis in various tumor types through NF-κB activation [273-275]. Moreover, several TLR polymorphisms are associated with cancer in a variety of tissues [272]. Thus, the modulating role of cPLA2α in TNF- and TLR-induced signaling reported in Papers II-IV may also be of relevance in inflammation-induced tumorigenesis. This hypothesis needs further investigation in appropriate model systems, and is an exciting direction for future studies.

Even though our understanding of the inflammatory processes and links between inflammation, proliferation and cancer development is substantial and rapidly increasing, many mysteries remain. Inflammation in the context of cancer is not always "bad". As always, the outcome of inflammation depend on balance; the cytokine in cancer can also contribute to driving the pathological process in a favorable direction [264]. At the beginning of the 20th century, the New York surgeon William Coley performed controversial research infecting cancer patients with "Coley's mixed toxins", an inflammatory-inducing bacterial extract (reviewed in [264]). Strikingly (if the published case histories are to be believed), he achieved impressive results. Today, we understand that "Coley's mixed toxins" were powerful activators of TLRs [35], and that the tumor-reducing effects were achieved by triggering a beneficial inflammatory response stimulating the immune system to target the tumor [264]. Several studies point to an anti-tumor effects of TLR signaling [272], and it is proposed that TLR-activation can induce a "re-education" of immune cells towards a tumor-suppressive phenotype through MyD88/NF-κβ dependent pathways [276]. The mechanisms by which inflammation can be shifted towards a beneficial path during cancer therapy are not clear, but the idea is intriguing and encourage for more research in the field. This yet again underscores the need to understand underlying signaling mechanisms, and the delicate balance between the many and pleiotropic mediators of inflammation.

4.4. cPLA2α inhibition as a therapeutic strategy

The development of TNF-blocking agents introduced a new era in RA treatment. However, the adverse effects including opportunistic infection, risk of recurrence and not to mention the significant number of non-responsive patients enlighten the need for new therapeutic targets; and cPLA2α is emerging as a potential candidate. In murine models, cPLA2α inhibition appears to be a promising therapeutic strategy in treating inflammatory arthritis [193, 194]. However, little is known about the detailed roles of cPLA2α and thus consequences of cPLA2α inhibition in the human body. However, thus far, increasing evidence from human cell culture studies, supplemented by the work presented in this thesis, suggest a central role for cPLA2α in modulating important

signaling comprising inflammatory, joint destructive and possibly proliferative processes also in human cells.

NSAIDs and COX2-specific inhibitors are effective in symptomatic relief in RA, however without affecting disease progression. COX2 inhibitors were developed to avoid the gastrointestinal adverse effects associated with long-term NSAID use. However, COX2-specific inhibitors turned out to display an increased incidence of myocardinal infarction and stroke [277], possibly due to a skewed TBX2/PGI2 ratio [278]. Both TBX2 and PGI2 are metabolized from AA downstream cPLA2α action, and PLA2 inhibition is as might be expected shown to decrease TBX2 biosynthesis [279]. In a patient with genetic mutations in the PLA2G4A gene [280], the TBX2/PGI2 ratio seemed to be skewed in a beneficial direction compared to healthy control patients [280], thus possibly indicating a favourable effect of targeting cPLA2α compared to selective COX2 inhibition. This hypothesis however, is not yet experimentally tested. Another example emphasizing the complexity in COX2-mediated lipid signaling is described by Gilroy *et al*. In a pleurisy rat model, they found an early peak in COX2 protein associated with maximal PGE2 synthesis during the onset of inflammation. However, at a later time-point, another, more powerful peak in COX2 protein was detected, coinciding with resolution of inflammation, decreased PGE2 synthesis and increased levels of the anti-inflammatory PGD2 and PGJ2. Inhibition of COX enzymes reversed this response, inhibiting early inflammation, but exacerbating inflammation at a later time point, possibly through inhibition of PGD2 and PGJ2 synthesis thus acting to inhibit resolution of inflammation. It is furthermore documented that different prostaglandins derived downstream COX2 activity can affect NF-κB in opposite ways; PGE2 and its analogs activate, while PGJ2 inhibits NF-κB activation. Thus, the balance between the different lipid metabolites is of vital importance [281]. It remains to be elucidated how cPLA2α inhibition affects the composition of lipid mediators in general, and whether cPLA2α inhibition provides a more balanced effect on COX-derived prostanoids. These are important questions to address in future studies.

Several pathways are proposed as potential future targets in RA therapy including MAPK signaling [282] and NF-κB activation [215]. However, these pathways orchestrate important processes in addition to pathologic inflammation, thus the

challenge lies in directly targeting pathological inflammation, permitting beneficial signaling necessary for a "healthy" immune response and homeostasis [215, 282]. Anti-TNF therapies are also, as previously mentioned, linked to adverse effects associated with a global suppression of the immune response [25]. The above-mentioned cPLA2α deficient patient [280] displayed platelet dysfunction and multiple recurrent small ulcers, implying the potential harmful consequences of a too drastic reduction in cPLA2α activity, which needs to be considered when considering safety and effectiveness of therapeutic cPLA2α inhibition. A general observation from the results presented in Papers II-IV is the normalizing effect of cPLA2α inhibition in response to pro-inflammatory stimuli; AA release and PGE2 levels were not short-circuited, but reduced to unstimulated control levels thereby possibly permitting basal house-hold activities. The same tendency was observed concerning gene transcription and protein production as cPLA2α inhibition served to reduce, not obliterate the expressions. Our results rather indicate a dampening effect of cPLA2α inhibition in multiple pro-inflammatory signaling pathways acting to normalize downstream cellular consequences. This may represent a favourable feature of cPLA2α as a therapeutic target as the inflammatory response depends on a delicate balance between many and often highly pleiotropic mediators. A slight shift in their composition may contribute to direct the inflammatory response in favor of resolution and repair. However, it remains to see if this hypothesis applies in animals or eventually in the human body, an exciting question for future research.

5. Conclusions

The overall objective of this work was to investigate the role of cPLA2α and lipid messengers in molecular mechanisms involved in chronic inflammation. Our results were also interpreted in light of evaluating cPLA2α as a future therapeutic target in inflammatory disease, RA in particular. In **Paper I**, we found PAF to be a regulator of keratinocyte proliferation, migration and eventually re-epithelialization, thus promoting wound healing rather than inflammation. In **Paper II**, we found cPLA2α to act as a regulator of synoviocyte TNF-induced signaling modulating the production of pro-inflammatory and joint destructive effectors. In **Paper III**, the regulatory role of cPLA2α was found to comprise TLR2-induced signaling. This signaling was elucidated in more detail in **Paper IV**, where cPLA2α, activated by MAPKs p38 and ERK1/2, was found to regulate TLR2-induced NF-κB activation and subsequent IL-6 generation, possibly through COX/PGE2-dependent pathways.

Taken together, our findings demonstrate the central role of cPLA2α, possibly via regulating PGE2 levels, and PAF in modulating important signaling cascades promoting inflammation and proliferation. Our results support a future role for cPLA2α as therapeutic target in treating rheumatoid arthritis and other chronic inflammatory conditions. Moreover, the role of cPLA2α in regulating (hyper)proliferation and possibly tumorigenesis is exciting for future studies.

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Paper I

Paper II

Cytosolic Phospholipase A2 Regulates TNF-Induced Production of Joint Destructive Effectors in Synoviocytes

Randi M. Sommerfelt☯, Astrid J. Feuerherm☯, Kymry Jones¤, Berit Johansen*

Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway

Abstract

Introduction: Rheumatoid arthritis (RA) is an inflammatory disease of the joint characterized by chronic synovitis causing pain, swelling and loss of function due to destruction of cartilage and bone. The complex series of pathological events occurring in RA is largely regulated via excessive production of pro-inflammatory cytokines, the most prominent being tumor necrosis factor (TNF). The objective of this work was to elucidate possible involvement of group IVA cytosolic phospholipase A2 (cPLA2α) in TNF-induced regulation of synovitis and joint destructive effectors in RA, to evaluate the potential of cPLA2α as a future therapeutic target.

Methods: The involvement of cPLA2α in tumor necrosis factor (TNF)-induced intracellular signaling cascades in synoviocytes (synovial fibroblast-like cells) was analyzed by arachidonic acid (AA) release assay, synoviocyte enzyme activity assay, gene expression analysis by real-time PCR and ELISA immunoassay for the detection of prostaglandin E2 (PGE2), interleukin 8 (IL8) and stromelysin-1 (MMP3), respectively.

Results: Inhibitors of cPLA2α enzyme activity (AVX002, ATK) significantly reduced TNF-induced cellular release of AA, PGE2, IL8 and MMP3. This reduction was evident both at transcriptional, protein or metabolite levels. Interestingly, cPLA2α inhibition affected several key points of the arachidonyl cascade; AA-release, cyclooxygenase-2 (COX2) expression and PGE2 production. Furthermore, the results suggest that cPLA2α is subject to transcriptional auto-regulation as inhibition of cPLA2α resulted in reduced PLA2G4A gene expression in TNF-stimulated synoviocytes.

Conclusions: cPLA2α appears to be an important regulator of central effectors of inflammation and joint destruction, namely MMP3, IL8, COX2, and PGE2. Decreased transcription of the PLA2G4A and COX2 genes in response to cPLA2α enzyme inhibition further suggest a self-reinforcing effect of cPLA2α inhibition in response to TNF. Collectively, these results support that cPLA2α is an attractive therapeutic target candidate as its inhibition reduces the production of multiple key pro-inflammatory factors involved in RA pathogenesis.

* E-mail: berit.johansen@ntnu.no

☯ These authors contributed equally to this work.

¤ Current address: Department of Psychiatry, New York University School of Medicine, New York, New York, United States of America

Introduction

Rheumatoid arthritis (RA) is an auto-immune and systemic inflammatory disease affecting 0.5-1% of the population, worldwide. In RA, chronic synovitis causes pain, swelling and loss of joint function due to degradation of cartilage and bone erosion [1]. Activated fibroblast-like synoviocytes (FLS) in the inflamed synovium are important contributors to arthritis through supranormal production of prostanoids, cytokines, chemokines, matrix degrading enzymes, angiogenic factors and adhesion molecules, thus perpetuating inflammation and joint destruction [2]. A key mechanism in the destructive signaling loop of RA is a dysregulation of the level of the pro-inflammatory cytokine tumor necrosis factor (TNF) [3,4]. TNF is overexpressed in RA synovium where it elicits a variety of biological effects on inflammation and immunity including modulation of gene expression and inflammatory joint destruction [5].

Phospholipase A2 (PLA2) enzymes release unsaturated fatty acids such as arachidonic acid (AA) by hydrolysis of the *sn*-2

ester bond of membrane glycerophospholipids. The arachidonyl specific group IVA cytosolic PLA2 enzyme (cPLA2α) encoded by the PLA2G4A gene is a major contributor to the elevated levels of AA in inflammation [6,7]. cPLA2α activity is regulated at many levels; by increased intracellular Ca^{2+} levels in response to pro-inflammatory stimuli, by binding to lipid second messengers, by phosphorylation induced by kinases, and by *de novo* gene transcription [8–10]. Following cPLA2α activation, the released AA is enzymatically metabolized to bioactive eicosanoids including prostaglandins, thromboxanes, lipoxins and leukotrienes [11]. Prostaglandin E2 (PGE2) is synthesized from AA through the cyclooxygenase (COX) pathway and is generally recognized as a potent lipid regulator of active inflammation [12]. The beneficial anti-inflammatory effect of reducing PGE2 synthesis is well recognized, and as such, non-steroidal anti-inflammatory drugs (NSAIDS) targeting the COX enzymes are widely used for symptomatic relief in RA [13]. However, long term use of NSAIDS has adverse effects e.g. affecting the gastrointestinal- and cardiovascular system and bone homeostasis [14–16]. The development of TNF-blocking agents has revolutionized the treatment of RA-patients and TNF-blockers are frequently used in RA therapy. However, approximately one-third of patients do not respond successfully to treatment [17]. Anti-TNF therapies are also under scrutiny following reports of malignancies, serious infections and long-term safety concerns [18,19]. Therefore, a search for alternative therapeutic targets is of great interest.

Several lines of evidence point to a role for cPLA2α in arthritis and inflammation, although the exact mechanisms of how cPLA2α regulates disease activity is not fully elucidated [7,20–23]. The aim of this study was to investigate the involvement of cPLA2α in joint and bone-destructive signaling in human synoviocytes. We identified cPLA2α as a regulator of TNF-induced expression of key players in RA pathology involved in bone and cartilage destruction, angiogenesis and neutrophil recruitment, namely stromelysin-1 (matrix metalloproteinase 3, MMP3), interleukin 8 (IL8), COX2 and PGE2. Furthermore, our results suggest that cPLA2α is subject to auto-regulation as inhibition of cPLA2α activity leads to reduced expression of PLA2G4A mRNA in response to TNF. Hence, our results support the comprehension that cPLA2α may be a major contributor to synovitis and joint destruction in RA, and therefore a potent therapeutic target candidate.

Materials and Methods

Reagents

Recombinant human TNF was from R&D systems (Abingdon, UK). Arachidonyl trifluoromethyl ketone (AACOCF3, ATK) was from Enzo Life Sciences (Farmingdale, NY, USA). PBS was from Oxoid (Basingstoke, Hampshire, UK). [3H]-arachidonic acid ([3H]-AA), and liquid scintillation cocktail Ultima Gold were from NEN Perkin Elmer (St. Louis, MO, USA). Leupeptin and pepstatin were from Roche Molecular Biochemicals (Indianapolis, USA). M-MLV reverse transcriptase, dNTPs and DTT were from Invitrogen (St. Louis, MO, USA). Random hexamer primers and RNAsin were from

Promega (Madison, WI, USA). DNAse- and RNAse-free water was from VWR (Pennsylvania, USA). RNeasy® minikit was from Qiagen (Valencia, CA, USA), ELISA kits for PGE2, IL8 and MMP3 were from Cayman Chemicals (Ann Arbor, MI, USA), Bender Medsystems (Vienna, Austria) and RayBiotech (Norcross, GA, USA), respectively. AVX002 was provided by Avexxin AS (Trondheim, Norway) and synthesized by Synthetica AS (Oslo, Norway). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

The human synovial sarcoma derived cell line SW982 was purchased from ATCC (London, UK). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 0.1 mg/mL gentamicin and 0.3 mg/mL L-glutamine at 37°C with 10% CO_2. Experiments were performed at 3 days post-confluence following overnight serum deprivation in serum-free DMEM. When inhibitors were applied, cells were pretreated for 2 hrs before stimulation with TNF (10 ng/mL).

[3H]-arachidonic acid release assay

Cells were labeled for 18 hrs with [3H]-AA (0.4 µCi/mL) in serum-free DMEM before experimental treatment. [3H]-AA release was analyzed in triplicates as previously described [24]. The results shown are released [3H]-AA in supernatants relative to total [3H]-AA incorporated into the cells. IC50 values for inhibitors were calculated as mean ± SD of at least 3 independent experiments.

Assay of cellular cPLA2α enzyme activity

SW982 synoviocytes were serum starved over-night before stimulation with TNF (10 ng/mL, 6 hours). Cells were lysed and 200 µg of total protein was analyzed for cPLA2α activity as described [24–27]. Bromoenol lactone (25 µM) and dithiothreitol (2.36 mM) were included in all reactions to inhibit activity of iPLA2 and sPLA2 [27].

Real-time reverse-transcription polymerase chain reaction (RT²-PCR)

Total RNA was isolated using RNeasy® minikit (Qiagen) according to kit protocol. RNA concentrations and integrity was monitored by NanoDrop spectrophotometric measurement (NanoDrop Technologies Inc. Wilmington DE, USA) and total RNA (1 µg) was reverse transcribed as described in [24]. Specific primers for IL8, MMP3, COX2, PLA2G4A and GAPDH with the following sense and antisense primers, were used in standard real-time RT-PCRs, with SYBR Green as fluorescence reporter: IL8, 5´-GACATACTCCAAACCTTTCCAC-3´ and 3´-CTTCTCCACAACCCTCTGC-5´, MMP3, 5´-TGATGAACAATGGACAAAGGATAC -3´ and 3´-CTGTGAGTGAGTGATAGAGTGG-5´, COX2, 5´-GGGGATCAGGGATGAACTTT -3´ and 3´-TGGCTACAAAAGCTGGGAAG -5´, PLA2G4A, 5´-CATGCCCAGACCTACGATTT -3´ and 3´-CCCAATATGGCTACCACAGG -5´, GAPDH, 5´-

CATCAAGAAGGTGGTGAAGCAG-3′ and 3′-TGTAGCCAAATTCGTTGTCATACC-5′. Cq values for each amplification curve were calculated by the Mx3000P software (Stratagene). Fold changes in mRNA expression and statistical analysis were calculated by the REST 2009 software [28] with mean PCR reaction efficiencies calculated by the LinRegPCR software [29] using GAPDH as reference gene.

Enzyme-linked immunosorbent assay (ELISA)

ELISA analyses were performed according to their respective kit protocols. The read-out for all ELISAs was carried out with a Multiscan plate reader (Ascent Labsystems). The corresponding Ascent software for Multiscan, Version 2.4.1 was used to obtain the data. Mean estimated IC50 value for PGE2 production was calculated from 3 independent experiments.

Statistical analysis

For AA release and ELISA analysis, statistical analyses were performed in SPSS Statistics 20 using one-way ANOVA at 95% confidence level in conjunction with Tukey HSD test. For real-time PCR data, statistical analysis was performed by the REST 2009 software [28]. Differences were considered significant at $p \leq 0.05$.

Results

TNF is a potent inducer of joint destructive regulators in synoviocytes

MMP3, IL8 and PGE2 are important regulators of inflammation and joint destruction in RA [12,30,31]. Matrix metalloproteinases (MMPs) are main contributors to RA cartilage destruction and the levels of several MMP subgroups, including MMP3, are elevated in RA synovial fluid [30,32]. The chemokine IL8 is overexpressed in RA synovium and acts as an angiogenic factor and chemoattractant for neutrophils thereby maintaining persistent migration of inflammatory cells into the synovium [31,33], while PGE2 is a powerful inducer of inflammation [12]. As TNF is known to induce both IL8 and MMP3 [3,34], we first characterized basal and TNF-induced gene expression by real-time PCR and protein by ELISA to justify the use of SW982 synoviocytes as a model for studying the TNF response. Indeed, TNF increased mRNA expression of MMP3 and IL8 by 45.6 ± 2.1-fold ($p \leq 0.01$), and 18.1 ± 3.7-fold ($p \leq 0.01$), respectively (Figure 1A and B). Correspondingly, TNF-induced protein expression was observed as indicated by a twofold increase in MMP3 levels from 5.5 ± 0.02 ng/mL to 10.5 ± 1.4 ng/mL ($p \leq 0.01$), and a fivefold increase in IL8 protein from 111.4 ± 15.6 ng/mL to 512.2 ± 28.2 ng/mL ($p \leq 0.01$, Figure 1C).

As we aimed to investigate involvement of cPLA2α in regulating TNF-induced expression of these metabolites, we further characterized the PLA2G4A gene expression in synoviocytes. We found the PLA2G4A transcript to be expressed in untreated cells, and further induced 4.8 ± 1.1-fold by TNF ($p \leq 0.02$, Figure 1D). Collectively, the SW982 synoviocyte model system was found suitable for investigating

potential involvement of cPLA2α in regulating TNF-induced signaling related to joint destructive processes occurring in the RA synovium.

AVX002 Efficiently Reduce AA Release and PGE2 Production

The AA metabolite PGE2 is recognized as a potent regulator of inflammation and the benefits of reducing pathological PGE2 levels are commonly accepted [12]. We aimed to investigate the effect of the recently described cPLA2α inhibitor AVX002 [25] on cellular AA release and total PGE2 synthesis in the synoviocyte model system. AVX002 dose-dependently inhibited the TNF-induced AA release with a mean estimated IC50 value of 0.9 ± 0.3 µM (Figure 2A). This estimate is based on 4 independent experiments. AVX002 alone modestly reduced basal AA release in a dose dependent manner; 5 µM AVX002 reduced basal AA release by 27%, 2.5 µM and 1.25 µM by 18%, and 0.63 µM by 13%. Furthermore, AVX002 was found to display long-lasting inhibitory effects evidenced by reduced TNF-induced AA release to $66 \pm 2\%$ of basal level following 72 hrs of TNF stimulation ($p \leq 0.05$) (Figure 2B). AVX002 inhibitory efficacy was compared to the widely used commercial cPLA2α inhibitor, ATK [35]. ATK reduced TNF-induced AA release in a similar fashion as AVX002 (Figure 2A), however with significantly lower efficacy (p=0.01) as indicated by the higher mean estimated IC50 value of 2.9 ± 0.8 µM.

Having shown that the inducing effects of TNF on AA release are normalized toward basal level by cPLA2α inhibitors, we next aimed to investigate if this finding was also reflected in the level of PGE2 production. By ELISA analysis, we demonstrated that production of PGE2 increased fivefold in response to TNF stimulation compared to basal level, from 52.6 ± 5.7 ng/mL to 265.5 ± 18.7 ng/mL ($p \leq 0.01$, Figure 2C). TNF-induced PGE2 production was dose-dependently reduced by AVX002 with a mean estimated IC50 value of 1.3 ± 0.3 µM. The IC50 estimate is based on three independent experiments. AVX002 alone also significantly decreased PGE2 production in unstimulated cells in a dose-dependent manner by 64% in 0.63 µM, 80% in 2.5 µM and 90% in 10 µM concentrations, compared to untreated control samples.

Since the metabolization of AA into PGE2 in response to TNF implies the involvement of the COX pathway, it was of interest to investigate the expression of the inducible COX2 enzyme. We show that in response to TNF, COX2 mRNA expression increased 9.0 ± 1.2-fold ($p \leq 0.01$), an induction that was significantly reduced by 50% following AVX002 treatment ($p \leq 0.03$, Figure 1D). Next, we investigated potential transcriptional regulation of the cPLA2α gene, PLA2G4A. We found that the fivefold induction by TNF was significantly reduced by 72% ($p \leq 0.03$, Figure 1D), suggesting that cPLA2α activity may be subject to auto-regulation in response to TNF stimulation. AVX002 treatment alone did not significantly affect basal transcription of either gene (results not shown). Hence, AVX002 served to normalize, not short-circuit gene expression thereby still allowing basal house-hold activities. Taken together, the inhibitory effect of AVX002 was evident at several regulatory levels and at different points in time. cPLA2α and COX2 enzyme activities are normalized towards basal activity
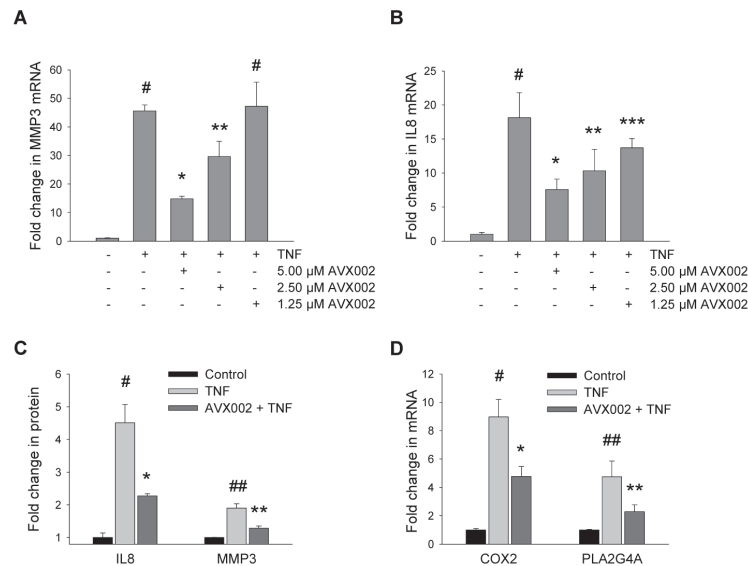
Figure 1. Inhibition of cPLA2α reduces TNF-induced expression of PLA2G4A, COX2, MMP3 and IL8. Fibroblast-like synoviocytes were treated with AVX002 (2 hrs) in indicated concentrations (A, B), 10 μM (C), or 5 μM (D), prior to TNF stimulation (10 ng/mL, 24 hrs). Total RNA was isolated and transcription of MMP3 (A) and IL8 (B), PLA2G4A and COX2 (D), was analyzed by real-time PCR as described in the Methods section. Amplification efficiency of all primer pairs were calculated by the LinRegPCR software and fold-change in gene expression compared to untreated samples was calculated by the REST 2009 software with GAPDH as reference gene. Supernatants were collected and analyzed by ELISA for MMP3 and IL8 protein (C) as described in the Method section (note starting point of Y-axis at 0.8). Data shown in all graphs are mean ± SEM (A, B, D) or mean ± SD (C) fold change compared to untreated samples for one representative of at least three independent experiments performed in duplicates. Significance is indicated as follows: A) #p ≤ 0.01 vs control; *p, **p ≤ 0.01 vs control and TNF-treated cells. B) #p ≤ 0.01 vs control; *p, **p, ***p ≤ 0.03 vs control and TNF-treated cells. C) IL8: #p ≤ 0.01 vs control; *p ≤ 0.01 vs control and TNF-treated cells. MMP3: ##p ≤ 0.01 vs control; **p ≤ 0.01 vs control and TNF-treated cells. D) COX2: #p ≤ 0.01 vs control; *p ≤ 0.03 vs control and TNF-treated cells. PLA2G4A: ##p ≤ 0.02 vs control; **p ≤ 0.03 vs TNF-treated cells.

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levels as monitored by means of AA release and PGE2 production and secretion. In parallel, COX2 and PLA2G4A gene expression are also reduced towards, but not below, basal levels. This suggests that the cPLA2α enzyme has an important regulatory role at several points in the AA cascade in synoviocytes.

AVX002 inhibits cPLA2α activity in cell lysates

To validate the inhibitory effect of AVX002 on cPLA2α in our model system, we investigated the potency of AVX002 to restrain cPLA2α enzyme activity in cell lysates from untreated and TNF-stimulated cells. Lysates of TNF-stimulated cells displayed an 80% increase in activity of cPLA2α compared to untreated cells as indicated by increased hydrolysis and release of ^{14}C-AA from phospatidylcholine visualized by TLC chromatography (Figure 2D). This stimulatory effect is consistent with the detected induction of AA release by TNF described above and validates the activation of cPLA2α in SW982 cells in response to TNF. Detection of cPLA2α activity

in lysates from unstimulated cells indicate a basal enzyme activity in synoviocytes, as previously described in primary rheumatoid synoviocytes [36,37], and correlates to the basal release of AA detected in unstimulated cells (Figure 2A). Furthermore, AVX002 efficiently inhibited activity of cPLA2α in lysates from unstimulated cells, as well as TNF-stimulated cells (Figure 2D). The capacity of AVX002 in inhibiting cPLA2α enzyme activity in cell lysates agrees with the previously reported effect on recombinant cPLA2α [25]. Together, these results support our interpretation that cPLA2α is activated by TNF in synoviocytes, and that AVX002 directly inhibits cPLA2α activity resulting in reduced downstream AA release and PGE2 production.

MMP3 expression is regulated by AVX002

To evaluate a potential role for cPLA2α in regulating destruction of cartilage in RA, we analyzed the effects of AVX002 on MMP3 expression. Indeed, AVX002 dose-dependently reduced TNF-induced MMP3 transcription with a
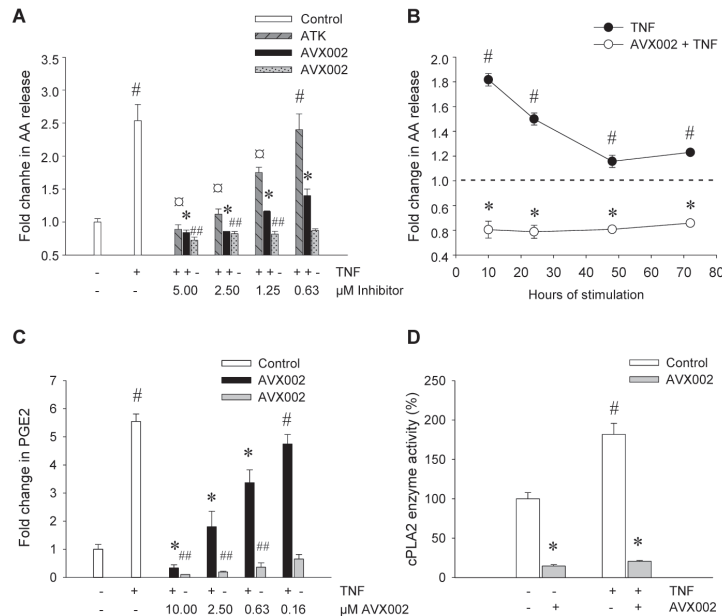
Figure 2. cPLA2α regulates TNF-induced AA release and PGE2 synthesis. SW982 synoviocytes were treated with either AVX002 or ATK (2 hrs) in indicated concentrations (A, C) or 5 µM (B) prior to stimulation with TNF (10 ng/mL) for 24 hrs (A, C) or indicated times (B). Analysis of released [3H]-AA (A, B) or PGE2 (C) was performed as described in the Methods section. Presented values are mean ± SD of TNF-induced release compared to untreated control samples in one representative of at least three independent experiments performed in duplicates (PGE2) or triplicates ([3H]-AA). AA release levels for untreated samples are indicated by a dash line in (B). Synoviocytes were stimulated with TNF (10 ng/mL) for 6 hours prior to lysis and detection of *in vitro* cPLA2α enzyme activity as described in the Methods section (D). Presented values are mean ± SD of *in vitro* cPLA2α activity (%) in one representative of at least three independent experiments performed in duplicates. Statistical significance is indicated as follows: A: #p≤ 0.01 when compared to untreated control values, *p ≤ 0.01 (AVX002) and ¤p ≤ 0.01 (ATK) when compared to TNF-stimulated control values. B: # p ≤ 0.05 when compared to untreated control values, *p ≤ 0.05 when compared to TNF-stimulated control values. C: #p ≤ 0.01, ##p ≤ 0.02 when compared to untreated samples, and *p ≤ 0.01 when compared to TNF-stimulated and control values. D: #p ≤ 0.03 when compared to untreated cell lysates, *p ≤ 0.02 when compared to untreated or TNF-stimulated cell lysates without inhibitor.

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maximum inhibition of 69% at 5 µM (p ≤ 0.01, Figure 1A). Basal MMP3 mRNA expression was not significantly affected by AVX002 alone (results not shown). Moreover, the TNF-induced twofold increase in MMP3 protein secretion was significantly reduced by AVX002 by 68%, from 10.5 ± 1.4 ng/mL to 7.1 ± 0.5 ng/mL (p ≤ 0.01, Figure 1C). These results suggest a role of cPLA2α in regulating TNF-induced MMP3 expression in human synoviocytes.

IL8 expression is regulated by AVX002

Next, the effect of AVX002 in regulating expression of the known neutrophil attractant IL8 was investigated by QPCR and ELISA immunoassay. AVX002 dose-dependently reduced TNF-induced up-regulation of IL8 transcription, with a maximum inhibition of 63% at 5 µM (p ≤ 0.03, Figure 1B). Basal

IL8 gene expression was not affected by AVX002 alone (results not shown). AVX002 also reduced TNF-induced IL8 protein secretion significantly by 65%, from 512.2 ± 28.2 ng/mL to 252.7 ± 62.8 ng/mL (p ≤ 0.01, Figure 1C). Accordingly, our results suggest that in human synoviocytes, TNF-induced IL8 expression may be regulated by cPLA2α.

Discussion

In RA, chronic inflammation and joint destruction is driven by excessive production of pro-inflammatory cytokines, chemokines and eicosanoids. In this study, by applying the chemical cPLA2α inhibitors AVX002 and ATK, we demonstrate that cPLA2α may be an important effector of TNF in intracellular signaling directly related to synovitis. Figure 3 summarizes the hypothesized involvement of cPLA2α in
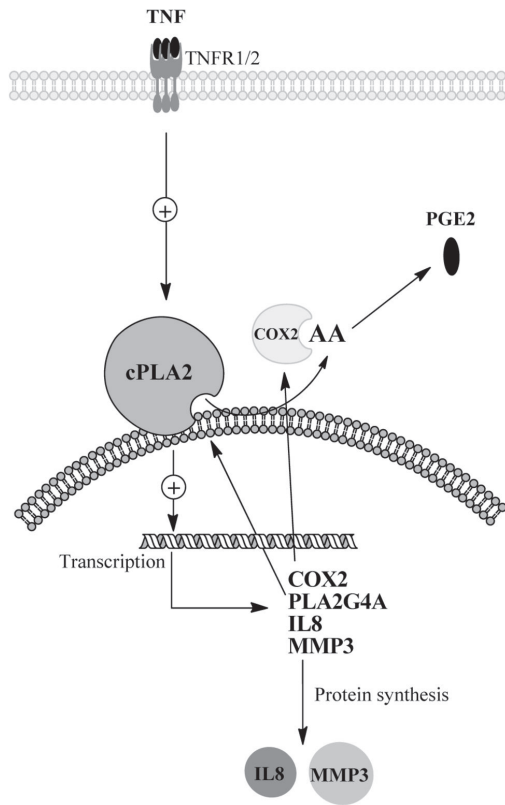
Figure 3. Proposed TNF-induced and cPLA2α dependent signaling in synoviocytes. TNF is a powerful inducer of inflammation and joint destruction in RA. In the SW982 synoviocyte model system, TNF induces activation of the cPLA2α enzyme to release AA from membranes, presumably through binding to its receptors TNF receptor 1 and TNF receptor 2 (TNFR1/TNFR2). AA released by cPLA2α is metabolized by COX2 to PGE2, a commonly recognized inducer of inflammation. The TNF-induced AA cascade can be self-reinforced by transcriptional regulation of COX2 and PLA2G4A genes. MMP3 and IL8 are central effectors in RA through cartilage destruction (MMP3), angiogenesis and attraction of immune cells (IL8). cPLA2α regulates TNF-induced expression of MMP3 and IL8 on transcriptional and protein levels. Consequently, cPLA2α functions to coordinate joint destructive and inflammatory processes in synoviocytes.
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regulating synoviocyte expression of key mediators of bone and cartilage destruction, angiogenesis and recruitment of immune cells, along with the availability of AA and subsequent PGE2 production.

Our results demonstrate that AVX002 efficiently and persistently reduces AA-release and hence the availability of substrate for pro-inflammatory eicosanoid production in synoviocytes (Figure 2). We have previously demonstrated that AVX002 is a potent inhibitor of recombinant cPLA2α enzyme activity *in vitro* [25] and that AVX002 efficiently inhibits PGE2 production in IL-1β stimulated rat mesangial cells. Here, we provide proof of principle that AVX002 potently inhibits cPLA2α as we for the first time demonstrate that AVX002 is an inhibitor of cPLA2α enzyme activity in synoviocytes (Figure 2D). Analogous effects of cPLA2α inhibition of enzyme activity, cytokine-induced AA release, PGE2 production and gene expression has previously been demonstrated with three other chemical inhibitors resembling AVX002 and ATK in chemical structure; MAFP, and the trifluoromethyl ketone analogue of EPA (EPACOCF3) [38–41]. Based on these results and the herein reported observations on the effects of AVX002 and ATK in our model system, we postulate that the observed effects of AVX002 are due to inhibition of cPLA2α enzyme activity.

The important role of PGE2 in propagating inflammation and pain is commonly recognized as reflected by the effective symptom relief of pain and stiffness by NSAID treatment in RA patients [12,13]. The highly pleiotropic PGE2 exhibits a wide range of biological actions [42] and is also proposed to be a central factor in bone and cartilage resorption in arthritis through regulation of osteoclast activity and expression of cytokines and MMPs in various model systems [43–45]. The versatile effects of PGE2 further include promotion of immune cell influx to inflamed tissue and angiogenesis [46,47]. As synoviocytes are important promoters of inflammation and joint destruction in RA, the reduced PGE2 production by AVX002 in these cells supports a key role for cPLA2α in RA pathogenesis. Our results further imply that reduced PGE2 synthesis in response to cPLA2α inhibitors may be self-reinforced through transcriptional regulation of the COX2 and PLA2G4A genes (Figure 1D). Transcriptional regulation of COX2 by cPLA2α has previously been shown in murine model systems [23,48] and our experiments suggest that this mechanism might be active also in human synoviocytes. The decrease in PLA2G4A mRNA in response to cPLA2α inhibition is to our knowledge a novel finding. It may suggest a feed-back loop from cPLA2α enzymatic activity through transcriptional regulation of the PLA2G4A gene in response to TNF in synoviocytes. Feed-back signaling where enzymatic activity of cPLA2α is required to regulate its own gene induction is previously reported in lung fibroblasts in response to IL-1β stimulation [9]. This possible auto-regulation in response to pro-inflammatory stimuli presents a potential self-reinforcing impact of inhibiting cPLA2α enzyme activity.

As degradation of cartilage and bone are major hallmarks of RA, disruption of these destructive processes represents a central therapeutic objective. MMP3, with its wide range of substrate specificity and ability to activate other MMPs, is essential in RA cartilage degradation [30] and correlate with disease activity and inflammation markers in RA patients [49]. We identify cPLA2α as a possible regulator of MMP3 expression in human synoviocytes (Figure 1A and C). This

correlates with reported findings from murine arthritis [23] suggesting that cPLA2α is a regulator of cartilage degradation in RA. Angiogenesis and the continuous influx of immune cells to the inflamed synovium are important processes driving the inflammation in RA joints. cPLA2α is proposed to be a regulator of neutrophil recruitment and inflammation in murine collagen-induced arthritis [22], and has been found to regulate expression of the chemotactic and angiogenic factor IL8 in human lung fibroblasts [50]. We show that cPLA2α enzyme activity might be involved in regulating IL8 production also in human synoviocytes (Figure 1B and C), emphasizing the potential biological relevance of cPLA2α in synovitis.

Given the complexity of TNF signaling networks, focusing on an intracellular therapeutic target downstream the TNF receptor may show reduced adverse effects compared to TNF-blocking therapy as many key host defense mechanisms are not targeted. Accordingly, a modulation of cPLA2α enzyme activity by specific cPLA2α inhibitors and subsequently normalizing downstream signaling may represent an alternative or supplement to current therapeutic strategies for RA treatment. Indeed, cPLA2α is expressed in RA synovium [51], and has been shown to play an important role in inflammation and in several animal models of arthritis [20–22]. Furthermore, inhibitors of cPLA2α including ATK and pyrroxyphene

ameliorate various inflammatory conditions including collagen-induced arthritis in mice [23,52]. Our results expand the understanding of cPLA2α as a possible regulator of inflammatory and joint destructive processes in human synoviocytes through regulation of MMP3, IL8 and PGE2.

Taken together, we demonstrate that cPLA2α may have an important role in regulating TNF-induced intracellular signaling in synoviocytes. Hence, our results suggest that cPLA2α may be involved in both inflammatory, angiogenic and tissue destructive processes and may hence be a promising therapeutic target to reduce inflammation and discomfort, pain, reduced functionality and mobility associated with RA.

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Author Contributions

Conceived and designed the experiments: AJF BJ RMS. Performed the experiments: RMS AJF KJ. Analyzed the data: RMS AJF BJ. Contributed reagents/materials/analysis tools: BJ. Wrote the manuscript: RMS AJF BJ.

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Paper III

Paper IV

Doctoral theses
in Biology

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos Zoology	Breeding events of birds in relation to spring temperature and environmental phenology
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos Zoology	Life aspects of two symparatic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation
1984	Eivin Røskaft	Dr. philos Zoology	Sociobiological studies of the rook *Corvus frugilegus*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos Zoology	Biochemical genetic studies in fish
1985	John Solem	Dr. philos Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains
1985	Randi E. Reinertsen	Dr. philos Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds
1986	Bernt-Erik Sæther	Dr. philos Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach
1986	Torleif Holthe	Dr. philos Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna
1987	Helene Lampe	Dr. scient	The function of bird song in mate attraction and territorial defence, and the importance of song

		Zoology	repertoires
1987	Olav Hogstad	Dr. philos Zoology	Winter survival strategies of the Willow tit *Parus montanus*
1987	Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway
1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987	Bjørn Åge Tømmerås	Dr. scient. Zoolog	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction
1988	Hans Christian Pedersen	Dr. philos Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care
1988	Tor G. Heggberget	Dr. philos Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure
1988	Marianne V. Nielsen	Dr. scient Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*)
1988	Ole Kristian Berg	Dr. scient Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.)
1989	John W. Jensen	Dr. philos Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth
1989	Helga J. Vivås	Dr. scient Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*
1989	Reidar Andersen	Dr. scient Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture
1990	Bengt Finstad	Dr. scient Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season
1990	Hege Johannesen	Dr. scient Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams
1990	Tor Jørgen Almaas	Dr. scient Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues
1990	Magne Husby	Dr.	Breeding strategies in birds: Experiments with the

			scient Zoology	Magpie *Pica pica*
1991	Tor Kvam		Dr. scient Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway
1991	Jan Henning L'Abêe Lund		Dr. philos Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular
1991	Asbjørn Moen		Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli		Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug		Dr. scient Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods
1991	Thyra Solem		Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund		Dr. philos Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism
1991	Nina Jonsson		Dr. philos	Aspects of migration and spawning in salmonids
1991	Atle Bones		Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen		Dr. scient Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher
1992	Anne Kjersti Bakken		Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen		Dr. scient Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen		Dr. philos Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks
1992	Arne Vollan Aarset		Dr. philos Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug		Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje		Dr. scient Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen		Dr. scient Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen		Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms

1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994	Morten Bakken	Dr. scient Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*
1994	Arne Moksnes	Dr. philos Zoology	Host adaptations towards brood parasitism by the Cockoo
1994	Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1994	Torbjørn Forseth	Dr. scient Zoology	Bioenergetics in ecological and life history studies of fishes.
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions
1995	Hanne Christensen	Dr. scient Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*
1995	Svein Håkon Lorentsen	Dr. scient Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition
1995	Chris Jørgen Jensen	Dr.	The surface electromyographic (EMG) amplitude as

		scient Zoology	an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport
1995	Vidar Moen	Dr. scient Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations
1995	Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae
1996	Ola Ugedal	Dr. scient Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines
1996	Christina M. S. Pereira	Dr. scient Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996	Jan Fredrik Børseth	Dr. scient Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics
1996	Gunnar Henriksen	Dr. scient Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region
1997	Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters
1997	Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming
1997	Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture
1997	Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997	Torgeir Nygård	Dr. scient Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors
1997	Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway
1997	Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry
1997	Rolv Lundheim	Dr.	Adaptive and incidental biological ice nucleators

		scient	
		Zoology	
1997	Arild Magne Landa	Dr. scient Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation
1997	Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*
1997	Jarle Tufto	Dr. scient Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997	Trygve Hesthagen	Dr. philos Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997	Trygve Sigholt	Dr. philos Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997	Jan Østnes	Dr. scient Zoology	Cold sensation in adult and neonate birds
1998	Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins
1998	Thor Harald Ringsby	Dr. scient Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998	Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998	Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity
1998	Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro
1998	Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach
1998	Bente Gunnveig Berg	Dr. scient Zoology	Encoding of pheromone information in two related moth species
1999	Kristian Overskaug	Dr. scient Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999	Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway
1999	Ingvar Stenberg	Dr. scient Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr.	A study of driftwood dispersal to the Nordic Seas by

		scient Botany	dendrochronology and wood anatomical analysis
1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr.	Biochemical impacts of Cd, Cu and Zn on brown

		scient Zoology	trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach

2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year

			period
2005	Tonette Røstelien	ph.d Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	ph.d Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	ph.d Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	ph.d Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	ph.d Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	ph.d Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	ph.d Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	ph.d Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	ph.d Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	ph.d Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	ph.d Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	ph.d Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	ph.d Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	ph.d Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	ph.d Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	ph.d Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	ph.d	Metal binding proteins and antifreeze proteins in the

		Biology	beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	ph.d Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	ph.d Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	ph.d Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007	Stig Ulland	ph.d Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	ph.d Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007	Roelof Frans May	ph.d Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	ph.d Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007	Julius William Nyahongo	ph.d Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	ph.d Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	ph.d Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	ph.d Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	ph.d Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	ph.d Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	ph.d Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	ph.d Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, (*Taeniopygia guttata*)
2008	Sølvi Wehn	ph.d Biology	Biodiversity dynamics in semi-natural mountain landscapes. - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	ph.d Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"

2008	Katarina Mariann Jørgensen	Dr.Scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	ph.d Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	ph.d Bilogy	*Arabidopsis thaliana* Responses to Aphid Infestation
2008	Jussi Evertsen	ph.d Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	ph.d Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	ph.d Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	ph.d Biology	Cost of rapid growth in salmonid fishes
2008	Line Johansen	ph.d Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	ph.d Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease
2009	Pål Kvello	ph.d Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	ph.d Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	ph.d Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches
2009	Zsolt Volent	ph.d Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	ph.d Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	ph.d Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	ph.d Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments -Focus on formulated diets
2010	Eli Kvingedal	ph.d Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	ph.d Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	ph.d Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. Tha Case of Serengeti Ecosystem, Tanzania
2010	Anton Tinchov Antonov	ph.d Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	ph.d	Population Ecology of *Eriophorum latifolium*, a

		Biology	Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	ph.d Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	ph.d Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	ph.d Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters
2010	Hans-Richard Brattbakk	ph.d Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	ph.d Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	ph.d Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	ph.d Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS:– The science of space experiment integration and adaptation to simulated microgravity
2011	Aline Magdalena Lee	ph.d Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics
2011	Christopher Gravningen Sørmo	ph.d Biology	Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in *Arabidopsis thaliana*
2011	Grethe Robertsen	ph.d Biology	Relative performance of salmonid phenotypes across environments and competitive intensities
2011	Line-Kristin Larsen	ph.d Biology	Life-history trait dynamics in experimental populations of guppy (*Poecilia reticulata*): the role of breeding regime and captive environment
2011	Maxim A. K. Teichert	ph.d Biology	Regulation in Atlantic salmon (*Salmo salar*): The interaction between habitat and density
2011	Torunn Beate Hancke	ph.d Biology	Use of Pulse Amplitude Modulated (PAM) Fluorescence and Bio-optics for Assessing Microalgal Photosynthesis and Physiology
2011	Sajeda Begum	ph.d Biology	Brood Parasitism in Asian Cuckoos: Different Aspects of Interactions between Cuckoos and their Hosts in Bangladesh
2011	Kari J. K. Attramadal	ph.d Biology	Water treatment as an approach to increase microbial control in the culture of cold water marine larvae
2011	Camilla Kalvatn Egset	ph.d Biology	The Evolvability of Static Allometry: A Case Study
2011	AHM Raihan Sarker	ph.d Biology	Conflict over the conservation of the Asian elephant (*Elephas maximus*) in Bangladesh
2011	Gro Dehli Villanger	ph.d Biology	Effects of complex organohalogen contaminant mixtures on thyroid hormone homeostasis in selected arctic marine mammals
2011	Kari Bjørneraas	ph.d Biology	Spatiotemporal variation in resource utilisation by a large herbivore, the moose
2011	John Odden	ph.d Biology	The ecology of a conflict: Eurasian lynx depredation on domestic sheep
2011	Simen Pedersen	ph.d Biology	Effects of native and introduced cervids on small mammals and birds
2011	Mohsen Falahati-	ph.d	Evolutionary consequences of seed banks and seed

	Anbaran	Biology	dispersal in *Arabidopsis*
2012	Jakob Hønborg Hansen	ph.d Biology	Shift work in the offshore vessel fleet: circadian rhythms and cognitive performance
2012	Elin Noreen	ph.d Biology	Consequences of diet quality and age on life-history traits in a small passerine bird
2012	Irja Ida Ratikainen	ph.d Biology	Theoretical and empirical approaches to studying foraging decisions: the past and future of behavioural ecology
2012	Aleksander Handå	ph.d Biology	Cultivation of mussels (*Mytilus edulis*):Feed requirements, storage and integration with salmon (*Salmo salar*) farming
2012	Morten Kraabøl	ph.d Biology	Reproductive and migratory challenges inflicted on migrant brown trour (*Salmo trutta* L) in a heavily modified river
2012	Jisca Huisman	ph.d Biology	Gene flow and natural selection in Atlantic salmon
2012	Maria Bergvik	ph.d Biology	Lipid and astaxanthin contents and biochemical post-harvest stability in *Calanus finmarchicus*
2012	Bjarte Bye Løfaldli	ph.d Biology	Functional and morphological characterization of central olfactory neurons in the model insect *Heliothis virescens*.
2012	Karen Marie Hammer	ph.d Biology.	Acid-base regulation and metabolite responses in shallow- and deep-living marine invertebrates during environmental hypercapnia
2012	Øystein Nordrum Wiggen	ph.d Biology	Optimal performance in the cold
2012	Robert Dominikus Fyumagwa	Dr. Philos.	Anthropogenic and natural influence on disease prevalence at the human –livestock-wildlife interface in the Serengeti ecosystem, Tanzania
2012	Jenny Bytingsvik	ph.d Biology	Organohalogenated contaminants (OHCs) in polar bear mother-cub pairs from Svalbard, Norway Maternal transfer, exposure assessment and thyroid hormone disruptive effects in polar bear cubs
2012	Christer Moe Rolandsen	ph.d Biology	The ecological significance of space use and movement patterns of moose in a variable environment
2012	Erlend Kjeldsberg Hovland	ph.d Biology	Bio-optics and Ecology in *Emiliania huxleyi* Blooms: Field and Remote Sensing Studies in Norwegian Waters
2012	Lise Cats Myhre	ph.d Biology	Effects of the social and physical environment on mating behaviour in a marine fish
2012	Tonje Aronsen	ph.d Biology	Demographic, environmental and evolutionary aspects of sexual selection
2012	Bin Liu	ph.d Biology	Molecular genetic investigation of cell separation and cell death regulation in *Arabidopsis thaliana*
2013	Jørgen Rosvold	ph.d Biology	Ungulates in a dynamic and increasingly human dominated landscape – A millennia-scale perspective
2013	Pankaj Barah	ph.d Biology	Integrated Systems Approaches to Study Plant Stress Responses
2013	Marit Linnerud	ph.d Biology	Patterns in spatial and temporal variation in population abundances of vertebrates
2013	Xinxin Wang	ph.d Biology	Integrated multi-trophic aquaculture driven by nutrient wastes released from Atlantic salmon (*Salmo salar*) farming
2013	Ingrid Ertshus Mathisen	ph.d Biology	Structure, dynamics, and regeneration capacity at the sub-arctic forest-tundra ecotone of northern Norway

and Kola Peninsula, NW Russia

2013	Anders Foldvik	ph.d Biology	Spatial distributions and productivity in salmonid populations
2013	Anna Marie Holand	ph.d Biology	Statistical methods for estimating intra- and inter-population variation in genetic diversity
2013	Anna Solvang Båtnes	ph.d Biology	Light in the dark – the role of irradiance in the high Arctic marine ecosystem during polar night
2013	Sebastian Wacker	ph.d Biology	The dynamics of sexual selection: effects of OSR, density and resource competition in a fish
2013	Ragnhild Pettersen	ph.d Biology	Identification of marine organisms using chemotaxonomy and hyperspectral imaging
2013	Angela Mwakatobe	ph.d Biology	Human-Wildlife Interaction in the Western Serengeti: Crop Raiding, Livestock Depredation and Bushmeat Utilisation
2013	Nina Blöcher	ph.d Biology	Biofouling in the Norwegian Salmon Farming Industry
2013	Cecilie Miljeteig	ph.d Biology	Phototaxis in Calanusfinmarchicus - light sensitivity and the influence of energy reserves and oil exposure
2013	Ane Kjersti Vie	ph.d Biology	Molecular and functional characterisation of signalling peptides of the IDA family in *Arabidopsis thaliana*
2013	Marianne Nymark	ph.d Biology	Light responses in the marine diatom *Phaeodactylum tricornutum*
2014	Jannik Schultner	ph.d Biology	Resource Allocation under Stress - Mechanisms and Strategies in a Long-Lived Bird
2014	Craig Ryan Jackson	ph.d Biology	Factors influencing African wild dog *(Lycaon pictus*) habitat selection and ranging behaviour: conservation and management implications
2014	Aravind Venkatesan	ph.d Biology	Application of Semantic Web Technology to establish knowledge management and discovery in the Life Sciences
2014	Kristin Collier Valle	ph.d Biology	Photoacclimation mechanisms and light responses in marine micro- and macroalgae
2014	Michael Puffer	ph.d Biology	Effects of rapidly fluctuating water levels on juvenile Atlantic salmon (**Salmo salar L**.)