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**Persistent Inflammatory  
Pathways in Rheumatoid  
Arthritis Despite Anti-rheumatic  
Treatment**

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Cover picture by Adrian Gheorghe.

The image illustrates the influence of anti-rheumatic therapies described in this thesis on the PGE<sub>2</sub> pathway.

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*To my family*



## ABSTRACT

The past years have witnessed tremendous progress in the treatment of rheumatoid arthritis, a chronic debilitating autoimmune disease mainly characterized by joint inflammation with progressive tissue destruction and loss of function. This condition affects 0.5-1% of the population, is associated with important co-morbidities and represents a heavy economical burden. New strategies, employing early and aggressive therapies with classical drugs or new agents, have resulted in impressive improvements in controlling disease activity. In some cases they even lead to clinical remission. Despite potent and efficient biological agents that specifically modulate distinct pathological pathways a large proportion of patients remain unresponsive to these therapies; drug-free remission is also difficult to achieve since attempting discontinuation of treatment usually results in disease flare.

In rheumatoid arthritis joints there is a constant activation of complex networks of cytokines and factors mediating immune interactions and inflammation, in which prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) are important players and contributors to pathogenesis. Our research aimed to investigate the synovial expression of enzymes controlling prostaglandin E<sub>2</sub> synthesis and degradation – cyclooxygenase (COX) 1 and 2, microsomal prostaglandin E<sub>2</sub> synthase 1 (MPGES1) and 15-prostaglandin dihydrogenase (15-PGDH) as well as enzymes involved in leukotriene synthesis, such as 5-lipoxygenase (LO) and 15-LO. In addition, we evaluated how traditional and new therapies influence these pathways, by analyzing enzyme expression before and after systemic treatment with tumor necrosis factor (TNF) antagonists, rituximab or methotrexate, as well as before and after intra-articular treatment with glucocorticoids. We also evaluated the *in vitro* effects of TNF antagonists and glucocorticoids on synovial fluid cells and that of methotrexate on synovial fibroblasts.

We demonstrated that synovial tissue from RA patients displayed an important expression of enzymes involved in the metabolism of PGE<sub>2</sub>, as well as 5-LO and 15-LO. MPGES1 and COX-2, the inflammation-inducible enzymes co-localized mainly in fibroblasts and macrophage-like cells and accounted for the local PGE<sub>2</sub> production. Intra-articular glucocorticoids significantly reduced all enzymes involved in the PGE<sub>2</sub> cascade – COX-1 and COX-2, MPGES1 and 15-PGDH, but also 5-LO, responsible for leukotriene formation. However, they did not influence the expression of 15-LO, an enzyme involved in the formation of both pro- and anti-inflammatory lipid mediators. Regarding the effects of TNF blockers, rituximab or methotrexate, they did not alter the expression profile of enzymes involved in PGE<sub>2</sub> metabolism despite showing clinical efficiency in improving disease activity. Although anti-TNF agents reduced the *in vitro* expression of MPGES1 and COX-2 in synovial fluid cells, the lack of effect *ex vivo* in biopsies emphasized once again the differences between synovial compartments and possibly the difficulty in mimicking the micro-environment at the site of inflammation *in vitro*.

In conclusion, this thesis demonstrates that potent anti-rheumatic drugs currently used in the clinic with good efficiency also leave inflammatory pathways unaffected, which may account for subclinical ongoing disease activity. Blocking the PGE<sub>2</sub> pathway by using MPGES1 inhibitors as combination therapy may show benefit in dampening ongoing local inflammation.

## LIST OF PUBLICATIONS

- I. **Effects of anti-rheumatic treatments on the prostaglandin E<sub>2</sub> biosynthetic pathway**  
Marina Korotkova, Marie Westman, Karina Roxana Gheorghe, Erik af Klint, Christina Trollmo, Lars Klareskog, Per-Johan Jakobsson  
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- II. **Prostaglandin E<sub>2</sub> synthesizing enzymes in rheumatoid arthritis B cells and the effects of B cell depleting therapy on enzyme expression**  
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- V. **Structural basis for induced formation of the inflammatory mediator prostaglandin E<sub>2</sub>**  
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## LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ACPA	Anti-citrullinated peptide antibody
ACR	American College of Rheumatology
BAFF	B cell activator factor of the TNF family
BLT	Leukotriene B <sub>4</sub> receptor
CIA	Collagen induced arthritis
COX	Cyclooxygenase
DAS	Disease activity score
DC	Dendritic cell
DMARD	Disease modifying anti-rheumatic drug
EULAR	European League Against Rheumatism
FLS	Fibroblasts-like synoviocytes
GC	Glucocorticoids
15-HETE	15-hydroxyeicosatetraenoic acid
HLA	Human leukocyte antigen
IFN $\gamma$	Interferon $\gamma$
IL	Interleukin
Ig	Immunoglobulin
LT	Leukotriene
LO	Lipoxygenase
LPS	Lipopolysaccharide
MAPEG	Membrane - associated proteins in eicosanoid and glutathione metabolism
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
M $\Phi$	Macrophages
MPGES1	Microsomal prostaglandin E <sub>2</sub> synthase 1
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OPG	Osteoprotegerin
PBMC	Peripheral blood mononuclear cells
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
PG	Prostaglandin
15-PGDH	15-prostaglandin dihydrogenase
PsA	Psoriatic arthritis
PWM	Pokeweed mitogen
RANKL	Receptor activator of nuclear factor kappa B ligand
SAC	<i>Staphylococcus aureus</i> Cowan strain I
SFMC	Synovial fluid mononuclear cells
TLR	Toll like receptors
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor



# FROM IMMUNITY TO INFLAMMATION AND BACK

## Innate and adaptive immunity

Immunity is the host defense system that enables an organism to fight and eliminate foreign pathogens through coordinate reactions of cells and molecules. Its two main components are the innate and the adaptive immune systems. Innate immunity confers the initial protection and is a primordial defense complex that allows rapid recognition and annihilation of pathogens. Furthermore, through recognition of an appropriate stimulus, it provides the signals necessary required by the adaptive immune system to mount a proper response. It is an early barrier to pathogens that acts immediately but does not generate lasting protective immunity. By contrast, the hallmark of adaptive immunity is antigen specificity and immunological memory. It is a system that responds gradually and relies on the innate system for initial fight with invading pathogens and further recruits and activates T and B lymphocytes, the adaptive immunity effector cells<sup>1</sup>.

Key components of the innate immunity are anatomical barriers, such as the epithelia in the skin, the gastro-intestinal and respiratory tracts, and professional phagocytes – neutrophils, macrophages and dendritic cells. Phagocytes can engulf microbes and destroy and eliminate them through a battery of degrading enzymes and free oxygen species. They achieve this by detecting pathogen- or damage- associated molecular patterns from invading microorganisms or injured structures. Dendritic cells are one bridge between innate and adaptive immunity via efficient antigen presentation. They capture the microbial antigen, migrate to peripheral lymphoid organs and display it via their major histocompatibility complex (MHC) class II molecules, in conjunction with co-stimulatory signals, to T cells. This initiates the early events in adaptive immune responses, whereby antigen specific T and B cells are formed. There is also a dual interaction between T and B cells providing potential positive feedback loops: on the one hand B cells mature and differentiate into memory cells or antibody-secreting plasma cells through cell-surface interaction and delivery of cytokines from T cells; on the other hand T cells are activated through antigen presentation and signals provided by B cells.

Upon encounter with the antigen, clonal expansion of antigen-specific T and B cells occurs. The specificity of T and B cells receptors is acquired by exposure to antigens through gene translocation and mutations that allow an increase in affinity and specificity.

The molecular motifs shared by various classes of microbes are recognized by innate immune cells through genetically - encoded pattern recognition receptors, of which toll-like receptors (TLRs) are a major family. Engaging of these receptors results in activation of inflammatory pathway cascades and provides signals to alert immune cells, subsequently inducing the activation program of adaptive immunity. One of their established ligands is the lipopolysaccharide (LPS) component of the microbial wall, also called endotoxin. It specifically activates TLR4 and through activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) induces the production of inflammatory mediators, cytokines and

bioactive lipids. Appropriate activation of the immune system and the inflammatory response are essential for defense against pathogens and for maintaining health.

## Inflammation and resolution

Inflammation is a protective response of the organism's immune system to injury or foreign challenge and is essential for restoration of tissue integrity and physiological homeostasis. It requires innate immunity and sometimes adaptive immune responses. The classical signs of inflammation – *rubor*, *tumor*, *calor*, *dolor* and *functio laesa* are the result of microcirculation events, activation of biological cascades and inflammatory cells and synthesis of a variety of cytokines, chemokines and lipid mediators, some with destructive potential. There are many mediators coordinating the initial events of acute inflammation, however many factors that drive inflammation also bring about its resolution<sup>2</sup>.

In normal conditions the acute phase of inflammation is followed by an active resolution process under the strict control of endogenous mechanisms. The latter involves not only suppression of pro-inflammatory genes, but also activation of pro-resolving mechanisms<sup>3</sup>, with reversal of vascular changes, inhibition of leukocyte migration and activation, as well as clearance of inflammatory cells by apoptosis and phagocytosis. A balanced control of the inflammatory and resolution reactions is important in maintaining the body's health state.

Biologically active fatty acids derivatives have essential roles in propagating inflammatory reactions but also in the resolution phase. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a lipid derivative that regulates a variety of cellular events in T and B lymphocytes, DC and macrophages and has a pivotal role in shaping and linking the innate and adaptive immune systems<sup>4</sup>. It is also an important mediator of inflammation, by inducing vasodilation, fever and pain. Leukotriene B<sub>4</sub> is a potent chemoattractant and activator of neutrophils with essential roles in inflammatory disorders. Conversely, lipoxins are potent anti-inflammatory agents active in nanomolar concentrations that inhibit migration of neutrophils to the inflamed tissue and promote recruitment of monocytes that will remove the cellular debris of the battle<sup>5</sup>. There is a time course formation of these mediators with switch from pro-inflammatory derivatives in the beginning to anti-inflammatory towards the resolution phase.

However, when the signals suppressing inflammatory reactions are defective, inflammation proceeds uncontrolled and ceases to be beneficial, resulting in pathogenic chronic disease states, such as rheumatoid arthritis. The driving force behind chronic inflammation is a perpetual activation of the adaptive immune system through cytokine feedback loops.

A remarkable feature of the immune system is its ability to react to a multitude of pathogens while avoiding attack on self structures. This is achieved through a thoroughly controlled process called immune tolerance by which the cells of the immune system get acquainted to the organism's own antigens and are "taught" to discriminate self from non-self. Immune tolerance takes place centrally in the thymus and bone marrow (central tolerance) and in periphery as a second fine-tuning process (peripheral tolerance). Failure in these mechanisms with subsequent dysregulation of immune reactions results in loss of tolerance against self structures and autoimmunity.

Autoimmunity is thus defined as an immune response towards self antigens. Cells of the innate and adaptive immune systems become activated and turn on unremitting inflammatory pathways, in such a way that the inflammation becomes chronic and complete resolution is very difficult to achieve.

Autoimmune conditions, such as rheumatoid arthritis, occur commonly and affect at least 1-2% of people in developed countries<sup>1</sup>. They feature chronic inflammation in one or more organs and they typically present with autoantibodies against organ-specific or ubiquitous antigens.

## RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic systemic inflammatory disease affecting 0.5- 1% of the population worldwide<sup>6</sup>, with a female preponderance and a peak incidence during the fourth decade of life<sup>7</sup>. Initially receiving its current name in the mid 19<sup>th</sup> century and being officially recognized as a distinct disorder even later, this disease has been around since several thousand years<sup>8</sup>. It affects primarily synovial joints, with chronic inflammation, subsequent destruction and gradual functional disability, and is associated with substantial systemic co-morbidities.

### *Clinical features, laboratory findings and diagnosis*

Rheumatoid arthritis usually has a gradual onset, starting with joint inflammation and tenderness, pain aggravated by rest and relieved by exercise, and morning stiffness. Fatigue, loss of appetite and low-grade fever may also be part of the general disease manifestations. Systemic features with pericarditis, pleuritis, lung fibrosis, vasculitis, rheumatoid nodules may co-exist at onset or become apparent during disease course. However, aggressive and efficient initial treatment has led to a lower frequency of extra-articular involvement<sup>9</sup>. Important co-morbidities in RA patients, such as risk increase for lymphoma and particularly cardiovascular disease, greatly reduce their life expectancy. In fact, cardiovascular diseases account for about half of the deaths in patients with RA<sup>10</sup>.

It is well-known that a large proportion of patients present autoantibodies in the blood. Rheumatoid factor is historically the most established autoantibody present in RA patients<sup>11</sup>. It is directed towards the Fc portion of immunoglobulins and its discovery led to the logical view that RA is an autoimmune disease. However, it has low specificity for the disease, since it is also present in healthy individuals (5%, frequency increasing with age) and patients suffering from other chronic inflammatory disorders<sup>12</sup>. More recently, anti-citrullinated peptide antibodies (ACPA) have demonstrated higher specificity for RA<sup>13</sup>. Both antibodies are clinically useful prognostic markers and predict a more aggressive, destructive course, in particular when associated with shared epitope<sup>14,15</sup>.

Since there is considerable heterogeneity in clinical manifestations, a set of criteria<sup>16</sup> was defined by the American College of Rheumatology (ACR) in 1987 to be used for differentiating RA from other inflammatory arthritides (Table 1).

*Table 1. The 1987 American College of Rheumatology classification criteria for RA*

1. Morning stiffness for at least 1 h
2. Arthritis in three or more joint areas with soft tissue swelling and fluid
3. Arthritis of hand joints (wrist, metacarpophalangeal or proximal interphalangeal joint)
4. Simultaneous involvement of the same joint areas on both sides of the body
5. Rheumatoid nodules
6. Rheumatoid factor
7. Typical radiographic changes on hand and wrist radiographs (erosions or bony decalcification)

Criteria 1- 4 should be present for at least 6 weeks. For classification as RA, at least four criteria must be fulfilled. (Adapted from Arnett et al. *Arthritis Rheum* 1988)

However, these criteria were developed to define established disease and provide a standard for recruitment into clinical trials<sup>17</sup>. The knowledge accumulated in the past years about pathogenesis and more specific serological markers as well as the success of early intervention with potent drugs has re-defined the strategy and goals for treatment and justified the need to identify patients at an early stage. Ideally, therapy should be instituted early in the disease development before erosions are apparent in order to prevent joint destruction<sup>18,19</sup>. Largely regarded as unsatisfactory for diagnosing purposes, these criteria were recently revised and new criteria have been proposed<sup>20</sup> (Table 2) as well as recommendation for pharmacological treatment.

*Table 2. The 2010 ACR/ EULAR classification criteria for RA*

	<b>Score</b>
<i>A. Joint involvement</i>	
1 large joint	<b>0</b>
2-10 large joints	<b>1</b>
1-3 small joints	<b>2</b>
4-10 small joints	<b>3</b>
>10 joints	<b>5</b>
<i>B. Serology</i>	
Negative RF and negative ACPA	<b>0</b>
Low positive RF or low positive ACPA	<b>2</b>
High-positive RF or high-positive ACPA	<b>3</b>
<i>C. Acute phase reactants</i>	
Normal CRP and normal ESR	<b>0</b>
Abnormal CRP or normal ESR	<b>1</b>
<i>D. Symptoms duration</i>	
<6 weeks	<b>0</b>
>6 weeks	<b>1</b>

Add scores in categories A-D. A score > 6/10 is needed for a definite RA diagnosis. For criteria B and C at least 1 test result is needed for the disease to be classified as RA (Adapted from Aletaha D. et al. *Ann Rheum Dis* 2010).

Disease activity score, often counted in 28 joints (DAS28), along with European League Against Rheumatism (EULAR) criteria are validated measures for disease activity, most commonly used to evaluate the patient's response to treatment in clinical trials<sup>21</sup>. These measures differ in that DAS evaluates the changes in diseases activity, while EULAR criteria take into account both the changes and the level of disease activity attained. DAS or DAS28 combine information from swollen joints, tender joints, the acute phase response – measured either as C reactive protein or erythrocyte sedimentation rate - and general health. The EULAR response criteria classify individual patients as non-, moderate, or good responders.

### Healthy and rheumatoid synovium

The healthy synovium is a thin membrane, two to three cells thick, divided in two anatomical and functional distinct regions, the intimal lining layer and the sublining layer. The lining layer is in contact with the joint space and the lubricating synovial fluid and is composed of macrophage-like synoviocytes (type A) and fibroblast-like synoviocytes (type B) in a loose extracellular matrix. It is an avascular structure lacking a basement membrane. In the sublining layer, few fibroblasts, adipocytes and scattered mononuclear cells are interspersed with vessels in a loosely organized connective tissue<sup>7</sup>.

In the established disease there is a marked proliferation of synoviocytes in the lining layer contributing to pannus formation, a structure with aggressive potential that drives the destructive process at the cartilage-bone junction. In the sublining, there is massive infiltration with mononuclear cells, especially macrophages, T and B cells and increased vascularization. In about one third of patients ectopic formation of lymphoid structures can be seen in the synovium<sup>22</sup>, some resembling germinal centers where maturation of B and T cells is thought to occur. Persistence of the antigen triggering the disease in the tissue is a critical element required for induction of these structures. Formation of lymphoid aggregates is not disease or tissue specific but rather the outcome of chronic inflammation<sup>23</sup>.

### Etiopathogenetic mechanisms

The etiology of the disease still remains unknown, but both genetic and environmental factors have emerged as having substantial influence in the development of RA<sup>19</sup>. A role for genes in the pathogenesis was revealed by twin studies in which the genetic component was shown to account for about 60% in the development of the disease<sup>24</sup>. Genetic evidence that the immunological response plays a critical role in the pathogenesis dynamics comes from association between RA occurrence and genes involved in immune regulation. The single most important genetic risk factor for RA is linked to the MHC class II locus<sup>25</sup>. The HLA DRB1 alleles code for a common aminoacid sequence – called the shared epitope - located in the antigen-binding region of the HLA molecule. A second identified genetic susceptibility gene in Caucasian population is PTPN22<sup>26</sup> which encodes a tyrosine phosphatase involved in T cell and B cell signaling<sup>27</sup>. These genetic associations, together with the presence of MHC-expressing antigen presenting cells<sup>28</sup> and T cells<sup>29</sup> in the rheumatoid synovium has

strengthened the argument for the contribution of MHC-dependent T-cell and B cell responses in the pathogenesis of the disease.

Accumulating data have indicated that cigarette smoking is the most important environmental factor increasing the risk of RA<sup>30</sup>, particularly in rheumatoid factor – positive and ACPA-positive patients<sup>31,32</sup>. Importantly, there is a major interaction between smoking and HLA DR risk alleles, with up to 20 times increased risk to develop the disease in genetically susceptible smoking individuals<sup>33</sup>.

The pathogenesis of RA is complex and yet far from being completely deciphered despite extensive research efforts. The evidence so far points to a pathogenic mechanism whereby in genetically susceptible individuals exposure to environmental insults (smoking) contributes to post-translation modifications in proteins (citrullination) that trigger dysregulated immune responses and breach of tolerance<sup>34</sup>. Additional un-identified trigger events may then contribute to joint localization, activation of immune responses, subclinical local inflammation and finally clinically apparent disease<sup>19</sup>. This series of events is particularly true for the subsets of RA patients displaying anti-CCP antibodies, whereas the ACPA negative subset has not been demonstrated to be associated to HLA locus or any established environmental factors. There are however studies suggesting different genetic and environmental profiles for ACPA-negative RA<sup>19</sup>. So far, accumulating data suggests the existence of two subsets of disease with different pathogenesis and prognostic factors, effects of environmental exposure and genetic susceptibility.

There are multiple mechanisms for synovial inflammation, with continuous activation of cells of the adaptive immune response by the multitude of cytokines present in the synovial compartment.

## Cellular network

Interaction and reciprocal regulation within an extensive network of cells characterizes the rheumatoid synovium. Below I will describe some features of the main cell populations.

### Monocytes/ macrophages

The role of monocytes and macrophages (MΦ) in the RA pathogenesis is underlined by their large number in the inflamed synovium, their broad pro-inflammatory and destructive potential and most importantly, by the correlation between radiographic progression of joint destruction and the degree of synovial macrophage infiltration<sup>35</sup>. Moreover, the degree of clinical improvement and response to treatment correlates well with a decrease in sublining MΦ for different therapeutic approaches<sup>36</sup>. Activated synovial MΦ may in fact represent a link between synovial inflammation and joint destruction.

There are two types of macrophages in the RA synovial tissue, the intimal lining macrophage-like synoviocytes and the sublining macrophages, that have migrated as monocytes from the circulation to the synovial compartment and are diffusely distributed throughout the synovium,. Both types have multiple and powerful biological functions underscored by their antigen presenting function, scavenging of cellular debris, chemotaxis through MΦ-derived cytokines and chemotactic factors and

formation of lipid mediators such as prostaglandins and leukotrienes<sup>37</sup>. They express several markers of the resident macrophage population including CD68, CD163 and CD14<sup>38</sup>. Owing to their plasticity, cells of the myelomonocytic lineage can be influenced by the cytokines/ growth factors balance in the microenvironment and can differentiate into several cell types that are critically involved in disease, as osteoclasts or dendritic cells. Upon TNF or IL-1 $\beta$  stimulation, synovial fibroblasts and activated T cells upregulate RANKL (receptor activator of nuclear factor  $\kappa$  B ligand) expression on their surface which can then engage its receptor (RANK) on the surface of monocytes and drive them into osteoclastogenesis<sup>39</sup>. Molecules relevant for osteoclast differentiation, such as macrophage colony stimulating factor, IL-17 and TNF, are abundant in the synovial tissue.

Macrophage activation and subsequent cytokine production is likely to occur through Toll-like receptors, known to promote and perpetuate inflammation. In RA, functional TLR2 and TLR4 are expressed in synovial and peripheral blood mononuclear cells. Their expression is upregulated by local cytokines. While being producers of key pro-inflammatory cytokines such as TNF, IL-1, IL-6, IL-12, IL-23, M $\Phi$  also secrete anti-inflammatory factors - IL-10 and IL-1RA. Most of the TNF and IL-1 in RA is produced by M $\Phi$  in the synovial membrane<sup>37</sup> and in particular at the pannus-cartilage junction. In addition, M $\Phi$  can secrete matrix metalloproteinases (MMP) 1, 2 and 9 and cathepsins that contribute to cartilage degradation and a series of chemokines that attract other immune cells<sup>37,40</sup>.

#### Fibroblasts

Fibroblast-like synoviocytes (FLS) in the rheumatoid synovium have different phenotypes in the lining and sublining regions. Intimal FLS, which are responsible for pannus invasion of neighboring structures, have unique invasive and aggressive features that differentiate them from other fibroblasts<sup>38</sup>. They display a unique phenotype resembling transformed cells which may be either an inherent feature of RA FLS or the result from an extensive exposure to cytokines. By expressing adhesion molecules they may also promote binding of mononuclear cells to the tissue. Since limited fibroblast proliferation was demonstrated in RA, the accumulation of FLS is thought to result from low apoptosis and increased survival promoted by the synovial milieu. Platelet-derived growth factor, basic fibroblast growth factor and in particular TNF and IL-1 may contribute to the synovial hyperplasia and activation. There is a paracrine/ autocrine network whereby activated macrophages produce pro-inflammatory factors that in turn activate FLS to produce their own arrays of mediators, sustaining positive feedback loops. They form chemokines and cytokines necessary for survival and activation of B cells and T cells (BAFF, IL-17) and massive amounts of matrix enzymes (MMPs, cathepsins) that degrade cartilage as well as their inhibitors. At the same time, FLS can express RANKL in response to inducing agents and contribute to formation of osteoclasts from their precursors. This indicates a central role for synovial fibroblasts in integrating and linking the inflammatory reaction with the destructive phase in the RA pathogenesis.

## T cells

The hypothesis that T cells are the major drivers of the dysregulated immune response and subsequent inflammatory effector mechanisms in RA is sustained by several observations: 1) T cells are found in high number in synovium<sup>29</sup>; 2) the disease is associated with certain MHC class II loci<sup>41</sup>; 3) the disease can be reproduced experimentally by transfer of T cells and many animal models of the disease are T cell dependent<sup>42,43</sup>.

In the synovial tissue, T cells are identified as CD3 positive and belong to one of the following subsets: CD4+ T helper cells, CD8+ cytotoxic T cells, or regulatory T cells, with a predominance of CD4+ cells<sup>38,44</sup>. The cytokines present in the environment dictate the direction of differentiation towards a particular T cell lineage. These pathways antagonize and cross-talk with each other through cytokines and are in certain cases mutually exclusive. The T helper (Th) phenotypes differ by their effector functions and are defined by a distinct pattern of cytokines formed. As such, the Th1 subset mediates cellular immunity and is defined by IFN $\gamma$  secretion; the Th2 is involved in humoral immunity and forms mainly IL-13 and IL-4, while Th17 is the newest member of this family identified through its signature cytokine, IL-17.

Data accumulated over the last years implicate Th17 cells as crucial promoters of autoimmunity in RA<sup>45</sup>. Earlier Th1 hypothesis stated that the rheumatic milieu promotes the polarization of T cell subsets towards a Th1 predominant phenotype<sup>46</sup>. There has been a shift in hypothesis during the last years and Th17 cells represent now the new paradigm of T cell contribution to RA pathogenesis.

Regulatory T cells are a subset of T cells detected in the synovial tissue and fluid of RA patients and their role is to maintain self-tolerance by regulation of immune responses and prevention of autoimmunity. However, these cells have impaired functions in RA and low suppressor ability<sup>47</sup> which can be restored by treatment with TNF inhibitors<sup>47,48</sup>.

## B cells

The B cell pathogenic role in autoimmune disorders has historically been attributed to their secretion of autoantibodies that would drive inflammatory cascades locally either in soluble form or as immune complexes. We know now that, apart from that, B cells are important antigen presenting cells and provide critical signals that regulate the activation and expansion of T cells in the synovium<sup>49</sup>. B cell-derived cytokines regulate dendritic cell activation and are important for the formation of ectopic lymphoid structures and their maintenance, as for instance lymphotoxin  $\beta$ <sup>50</sup>. In addition, B cells release cytokines (such as IL-6, IL-10) that provide feedback via interaction with T cells and macrophages. The interaction between B cells and T cells provides grounds for a potential vicious circle to sustain autoimmunity. A distinct subset of B cells producing IL-10 in mice were shown to have an immunoregulatory role in autoimmunity<sup>51</sup>, possibly by modulating macrophages and dendritic cells or restoring the cytokine balance<sup>52</sup>.

The lymphocyte infiltrate in RA tissue has several patterns of organization, from diffusely distributed or loosely aggregated to ordered structures resembling germinal



centers<sup>23</sup>. The formation of ectopic germinal centers seems not to be a specific feature of RA. It is present in several chronic inflammatory diseases and is thus the result of sustained inflammatory processes rather than being causative<sup>22</sup>. A number of factors produced by fibroblasts and dendritic cells, such as chemokine (CXC) ligand 12 and CXC13, BAFF, TNF and interferon (IFN)  $\gamma$  contribute to accumulation of B cells to the synovium and organization of these lymphoid structures<sup>52</sup>. In addition, B cells are critical in sustaining T cell activation in RA patients exhibiting ectopic lymphoid structures<sup>53</sup>. The evidence for pathological involvement of B cells in RA has come from experimental mouse models; autoantibodies seem to play a role in initiation of pathological events<sup>54</sup> while lack of B cells prevents the induction of arthritis and results in impaired T cells response in CIA<sup>55</sup>. In humans however, the key role of B cells in orchestrating the immune response in RA has become evident with the benefit achieved by B cell depleting therapy.

### Neutrophils

Neutrophils are the largest cell population in the synovial fluid but remarkably few of them are found in the tissue. They are the first to invade the synovial joint, followed by monocytes<sup>56</sup>, and contribute to the early pathogenesis phase by releasing reactive oxygen species and proteases that damage cells and tissues. By degrading cytokines or activating their precursors, they have the potential to regulate or amplify inflammation by recruiting and activating other immune cells. The impairment in apoptosis of synovial and peripheral blood neutrophils that can be seen early in the disease course can be reversed by methotrexate treatment<sup>57</sup>. The most powerful chemotactic agent for neutrophils is LTB<sub>4</sub><sup>58</sup>, found at high levels in synovial fluid<sup>59</sup>.

### Dendritic cells

Dendritic cells (DC) have essential roles in the orchestration of immune reaction in RA, since they are initiators and modulators of the immune response. Through their potent antigen presentation ability, they stimulate naïve T cells, direct effector cell functions and polarize the T cell repertoire towards the Th1, Th2 or Th17 phenotypes. Myeloid DC are considered especially important in promoting synovial inflammation and it is believed that they drive the activation of autoreactive T cells; plasmacytoid DC may have dual roles, in regulating antibody production and acting as tolerance inducers<sup>60</sup>.

## Inflammatory mediators

A large number of cytokines, chemokines, growth factors and lipid mediators promote constant recruitment and activation of cells sustaining synovitis and the processes leading to tissue destruction. This complex network of mediators is characterized by redundancy, with many cytokines having similar properties, and by activation of multiple signaling cascades with diverse biological effects<sup>61</sup>. There is a large number of monocyte- and fibroblast-derived mediators<sup>62</sup> which include IL-1, TNF, IL-6, IL-8, IL-10, IL-15, IL-18, GM-CSF, MIF, prostaglandins and leukotrienes, to name just a few. Lymphocyte-derived cytokines, such as IL-2, IFN $\gamma$ , IL-5, IL-13, but not IL-17, are found only at low levels in the synovial fluid, reflecting reduced T cell immune responses, possibly as a consequence of prolonged exposure of lymphocytes to

inflammatory stimuli<sup>63</sup>. A dominant role for TNF in driving the inflammatory cascade in rheumatoid synovial tissue became evident with the introduction and successful use of TNF blockers<sup>61</sup>. The concept emerging is that TNF is an early and important trigger of downstream inflammatory mechanisms and feedback loops within a network of immune and non-immune cells and cytokines.

TNF is released as a soluble cytokine after being cleaved from its membrane precursor from a variety of cells, mostly synovial macrophages and fibroblasts in RA. Binding to either of the two receptors – TNF receptor 1 (TNFR1) and TNFR2 leads to activation of NF- $\kappa$ B or to apoptosis, via two distinct pathways. TNFR1 is virtually expressed on all cell types whereas TNFR2 is inducible and mostly found on hematopoietic cells. TNF induces production of other mediators, such as IL-1, IL-6, INF $\gamma$ , PGE<sub>2</sub>; these in turn can induce regulatory positive or negative feedback loops<sup>64</sup>.

Major eicosanoids in the synovial fluid are PGE<sub>2</sub> and LTB<sub>4</sub>, although other prostanoids, leukotrienes and anti-inflammatory lipoxins are also present. They will be discussed in detail later in this book.

## Therapeutical strategies

There are several treatment options for RA, but despite significant therapeutic advances none has proven to cure the disease. Accumulating evidence over the past decade demonstrates that the earlier and most extensively the degree of disease activity is reduced, the better the outcomes will be.

### Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs provide symptom relief by blocking the formation of pro-inflammatory prostaglandins acting on the upstream enzymes, cyclooxygenases (COX) 1 and COX-2. Although they reduce pain and inflammation and improve joint mobility, there is no evidence that they slow down the disease progression. As such, they are prescribed as adjuvant along with more potent medication such as synthetic or biological disease-modifiers. Inhibition of constitutive COX-1 by non-selective NSAIDs may cause potential serious side effects. Even the selective COX-2 inhibitors are employed with caution due to increased cardiovascular events reported with their use<sup>65</sup>.

### Glucocorticoids

Glucocorticoids (GC) have been successfully used for decades in RA patients either systemically or as intra-articular injection to suppress disease activity or reduce local symptoms such as inflammation, swelling and pain. However, important side-effects, such as osteoporosis and diabetes, limit their long-term systemic use.

In practice, oral GC are usually used as a bridging therapy to control disease activity between the time a DMARD is initiated to the time it starts becoming clinically effective. Also, low-dose GCs are often added to DMARDs for their joint-protective effect<sup>18</sup>. Although they have a negative impact on bone mineral density with increased risk for fracture<sup>66</sup>, there is also evidence that low dose GC in combination with anti-rheumatic treatment yields better clinical outcomes and less radiographic damage<sup>67-69</sup>. This is most likely due to better control of the inflammatory status which per se has deleterious effects on the bone. Intra-articular GCs are used as efficient adjuvant

treatment<sup>70</sup> and their beneficial actions in RA are related not only to dampening inflammatory-related symptoms but also to protective bone effects by decreasing RANKL<sup>71</sup>. The latter is the most important mediator of osteoclast-dependent bone degradation and is induced in activated T cells and fibroblasts by TNF, IL-6, IL-17, PGE<sub>2</sub>, and possibly other factors<sup>72</sup>.

Their broad anti-inflammatory systemic effects are mediated through genomic mechanisms (activation or repression of gene transcription) and non-genomic mechanisms (independent of de novo protein synthesis)<sup>73</sup>. Repression of target genes by the GC-receptor complex is responsible for suppression of adhesion molecules expression, inflammatory cytokines, growth factors, prostanoids and other mediators, largely through inhibition of NF- $\kappa$ B-induced pro-inflammatory genes<sup>74,75</sup>. In addition, the GC-receptor complex can bind to specific nucleotide sequences termed GC-response elements and activate transcription of genes involved in resolution of inflammation, such as IL-10 and IL-1RA. Indirectly they can turn on mechanisms that limit inflammatory response through for example annexin 1 in innate and adaptive immune cells. Through non-genomic effects, GCs modulate the activation or responsiveness degree of cells involved in inflammatory reactions.

Disease-modifying anti-rheumatic drugs (DMARDs)

Disease-modifying anti-rheumatic drugs (DMARDs) are compounds able to retard progression of disease and slow down the joint damage. Apart from the traditional synthetic DMARDs that have been the standard of care in RA, the past two decades assisted to the emergence of a new class of potent drugs, so called biologics or biological DMARDs that comply to the definition of compounds halting disease progression and structural destruction<sup>64</sup>.

#### ◇ *Methotrexate*

Methotrexate is the mainstay therapy in RA and the first-line choice in the management of this disease<sup>76</sup>. It was originally developed to treat malignancies by antagonizing cellular folate and interfering with DNA synthesis. Although antiproliferative and immunosuppressive at high doses, there is increasing evidence from *in vivo*, *in vitro* and clinical data that in fact the anti-inflammatory effects of low-dose methotrexate – as seen in RA treated patients - are mediated through adenosine release. Other mechanisms that may be operating are suppression of T cell proliferation and increased apoptosis and modulation of cytokine formation and humoral responses<sup>77</sup>.

Methotrexate is an inhibitor of dihydrofolate reductase that forms tetrahydrofolate (THF) and as a consequence, of folate-dependent enzymes. Inhibition of dihydrofolate reductase decreases the availability of THF, interfering with DNA synthesis. At the same time, accumulation of upstream products results in increased AMP formation and subsequently adenosine. Supporting this notion, in a mouse air pouch model, methotrexate raised adenosine while reducing TNF levels in inflammatory exudates and reduced leukocyte trafficking<sup>78,79</sup>. Furthermore, the anti-rheumatic effects are diminished in patients treated with low doses methotrexate taking adenosine receptor antagonist<sup>80</sup>. Binding of adenosine to A<sub>2A</sub> and A<sub>3</sub> receptors is thought to mediate many of methotrexate's anti-inflammatory effects<sup>81</sup>, a concept arising from studies in

knockout mice. The beneficial effects in RA mediated by this pathway include inhibition of lymphocyte proliferation and production of TNF, IL-8, IL-12 and IFN $\gamma$ . It is known that methotrexate suppresses TNF production by T cells and macrophages<sup>82</sup> and IL-6 levels in RA patients, while IL-4 and IL-10 are increased in peripheral blood monocytes<sup>83,84</sup>, suggesting that methotrexate adjusts the balance between Th1 and Th2 cytokines. Moreover, by interfering with binding of IL-1 to its receptor<sup>85</sup> or reducing synovial IL-1 levels<sup>86</sup>, methotrexate may inhibit the cellular responses to IL-1, resulting in interruption of inflammatory circuits.

In RA patients, methotrexate is usually administered once weekly in doses ranging from 7.5 to 25 mg/week, orally or as a subcutaneous injection. Its efficacy as monotherapy and ability to increase the efficacy of biological therapy, combined with low toxicity profile makes it an anchor drug in RA.

Other DMARDs, including *leflunomide*, *sulfasalazine*, *injectable gold* and *hydroxychloroquine*, are recommended as second line approach if there is contraindication (or intolerance) to methotrexate<sup>87</sup>. In addition, immunosuppressive drugs such as *azathioprine*, *cyclosporine A* or *cyclophosphamide* have provided evidence of efficacy in refractory cases.

### Biological therapy

Biological drugs are a modern class of therapeutic agents derived from biologically active molecules and designed to modulate specific immune or inflammatory pathways.

#### ◇ *TNF blockers*

The introduction of TNF blockers in clinical use has revolutionized the treatment of RA to the point where it is now conceivable to aim at remission of disease rather than low disease activity. The great efficacy seen in many patients upon treatment, with marked amelioration of symptoms, reduced signs of inflammation and reduced systemic inflammatory parameters translate into a reduced disease activity and increased joint function<sup>64</sup>.

Perhaps the most important clinical benefit of TNF antagonists is the prevention of cartilage and bone destruction<sup>88-90</sup> and in some cases even reversal of erosions. The greatest efficacy is seen in combination therapy of TNF antagonist with methotrexate<sup>90</sup>, probably owing to complementary mechanisms of action. The mechanism by which TNF blockers inhibit erosion progression relies on reduced synovial macrophage number<sup>91</sup> and as a consequence reduced number of osteoclasts, diminished blood levels of MMPs<sup>92</sup>, regulation of RANKL and increased osteoprotegerin levels<sup>93</sup> – a decoy receptor for RANKL that blocks its action.

The dominant feature of anti-TNF action is diminished cellularity and leukocyte trafficking in the synovial compartment, likely occurring as a result of reduction in adhesion molecules, chemokines and cytokines such as IL-6, IL-1, IL-8, macrophage chemoattractant protein 1 and VEGF – a marker for increased angiogenesis in RA – both in synovial tissue, serum and blood<sup>61,64</sup>. Also, the apoptosis of monocytes/macrophages in synovial fluid may be an additional mechanism<sup>94</sup>. On the immune system level, TNF antagonists normalize the dysfunction in regulatory T cells by increasing their number and reversing their anergic phenotype<sup>47</sup>.

There are so far three TNF inhibitors in common clinical use and two more newly arrived on the market. *Infliximab* was the first out to be tested and is a human – mouse chimeric antibody containing the variable regions of a mouse anti-TNF antibody attached to the human constant anti-TNF antibody regions. It targets soluble and membrane bound TNF and is administered as an intravenous infusion. *Etanercept* is a soluble dimeric fusion construct consisting of the TNF receptor p75 bound to the Fc portion of a human IgG; it can bind soluble and membrane bound TNF as well as lymphotoxin (formerly known as TNF $\beta$ ) and is administered weekly as subcutaneous injection. *Adalimumab* is an entirely human IgG monoclonal antibody used once every second week as subcutaneous injection. In addition, two new TNF blockers have been approved during the last year, *golimumab* and *certolizumab pegol*; they seem to share similar efficacy profile and risks as the original members of this group<sup>95</sup>.

◇ *B cell depleting therapy*

*Rituximab* is a chimeric IgG<sub>1</sub> monoclonal antibody directed against CD20, a transmembrane molecule expressed on the B-lineage cells from the pre-B stage in the bone marrow throughout B cell development and differentiation in the periphery up to pre-plasma stage. Importantly, plasmablasts and antibody-secreting plasma cells do not exhibit this marker. It achieves B cells killing mainly through Fc $\gamma$  receptor antibody-dependent cell-mediated cytotoxicity<sup>96</sup>. However, marginal-zone B cells in the spleen and germinal centers B cells are resistant to depletion, probably due to protective niches where the antibody does not gain access<sup>97</sup>.

Despite complete depletion of circulating B cells in nearly all patients, lasting usually for about 4-6 months, there is a significant proportion of patients resistant to therapy that do not achieve significant clinical improvement<sup>98,99</sup>. It was shown that synovial B cells may persist in the tissue despite complete depletion in the circulation and persistence of plasma cells in the synovial tissue correlated with lack of response to treatment<sup>100,101</sup>. It is common that patients remain in remission for longer time than the depletion phase, but the relapse seems not to be related to peripheral re-population with B cells<sup>100</sup>. The observation that B cells in the synovial tissue are not depleted to the same extent as circulating B cells and that early re-emergence of memory B cells into circulation associates to poor clinical response<sup>102</sup> underscores the importance of depleting tissue autoreactive B cells.

Levels of RF and ACPA titers decrease slowly with treatment<sup>100</sup>, sometimes to undetectable levels, reflecting probably the decrease in short-lived plasma cells derived from targeted B cells that are responsible for autoantibody production<sup>96</sup>. In addition, reduction in follicular lymphoid structures size and number following treatment mirrors the decrease in synovial B cells and macrophages and reflects decreased synovitis<sup>103</sup>. The mode of action of rituximab suggested by recent studies presumably involves depletion of tissue autoreactive B cells, with several consequences - decreased autoantibody and immune complex formation, reduced cytokine production, followed by reduced macrophage load and decreased synovial inflammation<sup>104</sup>.

Newer B cell depleting drugs are in late stage clinical development; *ofatumumab* is a fully humanized anti-CD20 antibody expected to have similar efficacy to rituximab. *Belimumab* and *atacicept* target survival factors (BAFF and APRIL) or their receptors

involved in survival and differentiation of B cells. Clinical evaluation trials are ongoing to assess their efficacy and safety profiles.

There are a number of other biological drugs in clinical use: *anakinra* blocks IL-1 receptor and has some efficacy in RA, although far from the one achieved by TNF antagonists<sup>95</sup>. Both *tocilizumab* blocking the IL-6 receptor and *abatacept* targeting the co-stimulatory pathway for T cells show similar efficacy to TNF blockers; while *tocilizumab* possibly induces remission in a higher proportion of patients, *abatacept*-treated patients show fewer infections than *infliximab*<sup>105</sup>. They are usually used in cases where other biological therapies have failed and the recommendation is as for TNF inhibitors, to be associated with methotrexate for increased efficacy. Also, methotrexate prevents formation of anti-drug antibodies<sup>106</sup>.

Although still far from achieving the goal of inducing stable drug-free remission, studies on the immunological pathways targeted by anti-cytokine therapies and identification of tissue and serum markers associated with response or resistance to treatment provide insights into new and possibly more efficient drug targets.

## EICOSANOIDS

Eicosanoids are a class of lipid mediators with a wide variety of biological activities stemming primarily from arachidonic acid, a twenty-carbon fatty acid. The word eicosanoid is derived from the Greek *eicosi* meaning 20. The main classes of eicosanoids - prostaglandins, leukotrienes and lipoxins - are synthesized by cyclooxygenases and lipoxygenases from fatty acid-derived precursors. The first step in the synthesis of eicosanoids is catalyzed by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) that releases arachidonic acid (AA) from membrane phospholipids upon cell stimulation, usually by cytokines, growth factors, mechanical stress or other noxious stimuli.

The PLA<sub>2</sub> family comprises over 25 members grouped in three classes: secretory PLA<sub>2</sub>, calcium-dependent (also called cytosolic) and calcium-independent intracellular PLA<sub>2</sub>. They have diverse roles in host defense and inflammation, with some contributing as pro-inflammatory and other to ameliorate inflammation<sup>107</sup>.

### Cyclooxygenase pathway

Prostaglandins (PG) arise through sequential actions of cyclooxygenase (COX) enzymes which initially form an intermediate unstable PGH<sub>2</sub>, rapidly converted by terminal synthases to the bioactive lipid compounds, namely PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub> (often called prostacyclin) and tromboxane A<sub>2</sub><sup>108</sup>.

#### Prostaglandin E<sub>2</sub>

PGE<sub>2</sub> is the most ubiquitously produced prostaglandin under physiological and pathological conditions and regulates fundamental biological processes through broad spectrum effects. It is involved in regulation of immune responses, vascular pressure control, integrity of the gastro-intestinal tract mucosa, kidney perfusion and female reproduction<sup>109</sup>. Apart from its homeostatic roles, PGE<sub>2</sub> exerts key biological functions in inflammation, pain, fever regulation and tumorigenesis<sup>110</sup>.

PGE<sub>2</sub> exerts its functions by binding to one of its four receptors named EP1 through EP4, all G-protein coupled receptors (GPCRs) that constitute the prostanoid receptor family within the GPCR superfamily<sup>111</sup>. The main cellular transduction pathway in EP2 and EP4 involves signaling via rising intracellular cAMP, in EP3 via decrease in cAMP, while in EP1 is mediated through increase in intracellular calcium concentration<sup>111</sup>. The variable tissue distribution and cell localization of the receptors determine cell-specific effects of PGE<sub>2</sub> signaling depending on the local cellular environment.

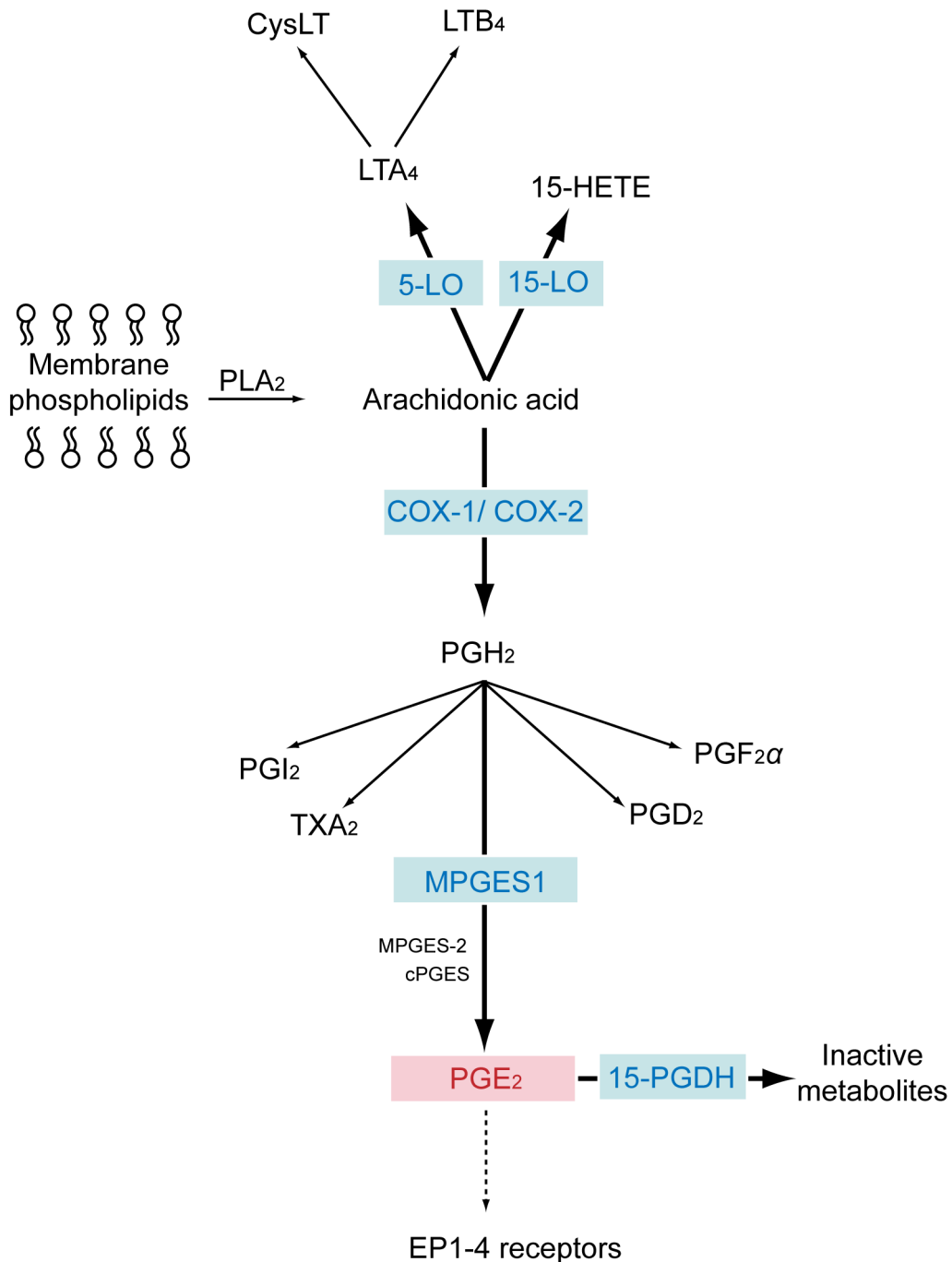


Fig. 1 Simplified version of the eicosanoid pathway with relevance to pathways investigated in my studies.

There are extensive studies on PGE<sub>2</sub> in acute and chronic inflammatory conditions. The ability to regulate the immune response at various levels, from cells involved in innate immunity to antibody synthesis makes PGE<sub>2</sub> an important contributor to immune-mediated diseases.

PGE<sub>2</sub> formation is highly increased during inflammatory conditions and it is fair to say that it mediates all signs of inflammation. Vasodilation with increased blood flow resulting in local swelling, heat and redness, peripheral nociception with hyperalgesia and in some cases promotion of tissue destruction can all result from PGE<sub>2</sub> release<sup>2,109</sup>.

PGE<sub>2</sub> is known to modulate the activity of antigen presenting cells (APC), such as dendritic cells and macrophages, and shape the outcome of the adaptive immune response by influencing the cytokine balance in the microenvironment<sup>4</sup>. Depending on the site of encounter, PGE<sub>2</sub> exerts stimulatory or inhibitory effects on DC. In periphery, PGE<sub>2</sub> activates and promotes migration of DC to the lymph nodes, but once there, it inhibits their maturation and antigen presentation ability. PGE<sub>2</sub> has been implicated in inhibition of T cell proliferation owing to decrease in IL-2 production<sup>112</sup> but also in modulation of T cell apoptosis<sup>113,114</sup>. In fact, it plays important roles throughout the life of T cells, from thymic selection to proliferation, cytokine secretion and apoptosis. In general, it was considered that PGE<sub>2</sub> may favor a Th2 response over Th1 by inhibiting IFN $\gamma$  and IL-2 secretion, while sparing IL-4 and IL-5 formation<sup>115</sup>. However, this concept arising from *in vitro* studies was challenged recently by investigations showing that physiologically low PGE<sub>2</sub> concentrations facilitate a Th1 response in mouse<sup>116</sup>. Also, PGE<sub>2</sub> can synergize with IL-23 facilitating Th17 cell expansion<sup>117</sup> and promotes accumulation of antigen-specific Th1 and Th17 cells in lymph nodes in mouse autoimmune encephalomyelitis, mainly through EP4 receptors<sup>118</sup>. Indeed, increasing focus on Th17 cells as drivers of autoimmune reactions has led to new insight into the role of PGE<sub>2</sub> to promote Th17 polarization of the immune response. PGE<sub>2</sub> regulates the cytokine expression pattern by DC and can bias the T cell repertoire towards Th1 or Th17 dominance<sup>115</sup>. Moreover PGE<sub>2</sub> mediates joint inflammation and damage in mouse collagen-induced arthritis (CIA) through IL-23/IL-17 axis<sup>119</sup>.

In B cells, PGE<sub>2</sub> assumes inhibitory roles in the lineage development, as mice treated with PGE<sub>2</sub> show fewer B cell precursors in their bone marrow<sup>120</sup>. In the mature B cell, PGE<sub>2</sub> has immunosuppressive roles and represses proliferation of B cells through the prostaglandin EP4 receptor gene<sup>121</sup>. It is known that activated, but not resting peripheral blood B cells can form PGE<sub>2</sub> through COX-2 upregulation<sup>122</sup>.

In activated macrophages, PGE<sub>2</sub> dampens production of TNF, IL-1 and IL-12 and increases IL-10<sup>123,124</sup>. On the other hand, LPS-stimulated macrophages are an important source of PGE<sub>2</sub>; there is also an autocrine feedback regulation whereby PGE<sub>2</sub> can upregulate COX-2 and its own formation<sup>124,125</sup>.

With relevance to rheumatoid arthritis, PGE<sub>2</sub> has a pivotal role in local bone remodeling and there are many studies underlining its role in bone-resorptive diseases<sup>126</sup>. Bone is a living tissue with osteoclasts removing old bone and osteoblasts replacing it with new one. Resorption is dependent on interaction between RANKL on osteoblasts, activated T cells or fibroblasts and RANK on osteoclast precursors<sup>72</sup>. In addition, osteoprotegerin (OPG) can prevent the formation of osteoclasts by binding to RANKL. There is not a clear consensus as to whether PGE<sub>2</sub> promotes anabolic or catabolic



processes in the bone and in fact the net effect may depend on the type of receptors locally present. Both EP2 and EP4 receptors mediate bone resorption in response to PGE<sub>2</sub> in mice, since lack of these receptors greatly reduces the osteoclastogenic response<sup>127,128</sup>. Cytokines such as IL-1 and IL-6 induce osteoclast formation via PGE<sub>2</sub><sup>126</sup> and conversely, PGE<sub>2</sub> stimulates the release of these same cytokines. There appears to be a synergistic interaction between PGE<sub>2</sub> and IL-1 or IL-6 culminating with RANKL production and OPG inhibition. Although in mice PGE<sub>2</sub> seems to promote bone degradation, studies in human reveal conflicting data. In human peripheral blood mononuclear cells (PBMC), PGE<sub>2</sub> inhibited RANKL/M-CSF-induced osteoclast formation, while in bone marrow cell cultures PGE<sub>2</sub> had an opposing effect<sup>126</sup>.

PGE<sub>2</sub> is found in large amounts in RA synovial fluid<sup>129</sup> and coordinates several mechanisms involved in the pathogenesis of rheumatoid arthritis. The synovial tissue is highly proliferative and relies on new vessel generation for oxygen supply. As such, PGE<sub>2</sub> stimulates the vascular endothelial growth factor (VEGF) production in fibroblasts<sup>130</sup> thereby contributing to angiogenesis. The destruction process in rheumatoid arthritis involves degradation of cartilage and bone. Important in contributing to cartilage destruction, PGE<sub>2</sub> mediates proteoglycan degradation in osteoarthritis cartilage extracts<sup>131</sup> and inhibits de novo collagen synthesis<sup>132</sup>. As pointed out earlier, PGE<sub>2</sub> contributes to skewing the immune response towards a Th17 phenotype in the synovial microenvironment further enhancing release of inflammatory cytokines and promoting bone degradation. *In vitro* stimulated synoviocytes mount an impressive response in PGE<sub>2</sub><sup>133</sup>, while synovial biopsies from RA patients are known to express enzymes of the PGE<sub>2</sub> biosynthesis pathway<sup>134</sup>.

EP4 receptor antagonist relieves joint pain and inflammation in animal model of arthritis<sup>135</sup>. Moreover, EP4 is the receptor responsible for mediating PGE<sub>2</sub> destructive potential in arthritis, since only the EP4 and not the other receptor knockout animals displayed reduced incidence of CIA, along with less cartilage and bone destruction compared to their wild-type littermates<sup>136</sup>. Also, macrophages isolated from mice lacking EP4 are less prone to produce TNF and IL-6<sup>136</sup>.

Fatigue, a common complaint of RA patients, is caused by proinflammatory cytokines acting on the blood-brain barrier and inducing brain formation of PGE<sub>2</sub>, which signals further to the central parts of the brain<sup>137</sup>.

Biosynthesis of PGE<sub>2</sub> is catalyzed by various PGE<sub>2</sub> synthesizing enzymes, either microsomal or cytosolic (MPGES1 and -2 or cPGES). MPGES1 is the major enzyme of the PGE<sub>2</sub> synthesis in inflammatory conditions and will be discussed in detail in the next chapter. MPGES-2 is constitutively expressed in many tissues, with high levels reaching in the brain, heart, muscle, liver and kidney<sup>138</sup>. Although cPGES is ubiquitously and constitutively expressed in most cells, pro-inflammatory stimuli in brain tissue were shown to increase its activity and expression<sup>139</sup>.

Prostaglandins leave cells and enter extracellular compartment driven by pH and membrane potential or through active PG transporters, proteins with broad tissue distribution<sup>140</sup>.

The local PGE<sub>2</sub> concentration depends on the balance between synthesis and degradation. Inactivation of PGE<sub>2</sub> is carried out by 15-prostaglandin dihydrogenase (15-PGDH). There are two types of 15-PGDH: type I (referred throughout this book to

as 15-PGDH) catabolizes specifically PG and other eicosanoids, while type II exhibits broader substrate specificity and is believed to have minor function in the catabolism of PGs<sup>141</sup>. In the next paragraphs I will focus on the main enzymes involved in synthesis and degradation of PGE<sub>2</sub>, namely MPGES1, COX and 15-PGDH, with particular emphasis on inflammatory arthritis conditions.

#### PGE<sub>2</sub> biosynthesis - MPGES1 and COX enzymes

Synthesis of PGE<sub>2</sub> is dependent on the amount of AA available and on the presence of its synthesizing enzymes, MPGES1 and COX. Of the two COX isoforms, MPGES1 is coupled functionally mostly with COX-2, as they also co-localize<sup>137,142</sup> and can be co-regulated in many cases. However, the promoter region of MPGES1 gene, unlike COX-2, displays few transcriptional elements typical to cytokine-induced genes<sup>143</sup>, indicating that MPGES1 and COX-2 have divergent mechanisms operating for regulation of their induction.

MPGES1 is an inducible, 16.5 kDa enzyme belonging to the MAPEG (membrane associated proteins in eicosanoid and glutathione metabolism) family which includes six human proteins<sup>144</sup>. The highest homology to MPGES1 is found in microsomal glutathione S-transferase 1 (MGST1). All members of the MAPEG family are 16-18 kDa integral membrane proteins, have a conserved six amino acid sequence and share similar hydropathy profiles. In addition to the two previously mentioned human proteins, the MAPEG family comprises also the human 5-lipoxygenase activating protein (FLAP), leukotriene C<sub>4</sub> synthase (LTC<sub>4</sub> synthase), MGST2 and MGST3. Within the cells, MPGES1 resides in the nuclear and endoplasmic reticulum membranes and on sub-cellular fractionation it is found in the microsomal fraction<sup>110</sup>.

COX enzymes are membrane bound proteins found as two isoenzymes; although their functional role is not absolute, COX-1 is a constitutive enzyme expressed virtually in all tissues of the body and its products serve homeostatic functions, such as gastroprotection and haemostasis<sup>108</sup>. Its gene promoter does not possess any elements reminding of an inducible gene; however modulation of COX-1 expression has been reported<sup>145</sup>. COX-2 is induced by cytokines and mitogens which increase transcription but also stability of mRNA<sup>146</sup>, resulting in increased protein expression. It largely accounts for the prostaglandins formed in inflammation and cancer, but it can also be constitutively expressed in brain and kidney<sup>147,148</sup>. In contrast to its housekeeping functions, COX-1 deletion does not result in a severe phenotype in mice under physiological resting conditions, while COX-2 deficient mice display altered functions in several organs, including kidney, heart and reproductive organs<sup>149</sup>.

Although constitutively expressed in a few human tissues, such as prostate, testis<sup>150</sup>, stomach, muscle tissue<sup>151</sup>, the main function of MPGES1 is to upregulate PGE<sub>2</sub> formation in pathologies associated with inflammatory response. LPS was the first to demonstrate co-induction of MPGES1 and COX-2 in rat peritoneal macrophages<sup>152</sup>. Other known MPGES1 inducers are IL-1 $\beta$ <sup>150</sup>, TNF, IL-6. Enzyme upregulation in response to inflammatory stimuli can be observed in fibroblasts, macrophages, osteoblasts, endothelial cells, smooth muscle cells, chondrocytes, and cardiomyocytes<sup>153,154</sup>. The molecular mechanisms responsible for MPGES1 induction can be different depending on cell type and activation stimuli, but common final

pathways may lead to activation of transcription factors such as NF- $\kappa$ B and Egr-1 (early growth response gene)<sup>155</sup>. Subsequently, a large number of genes involved in inflammatory responses are transcribed, among others MPGES1 and COX-2<sup>156,157</sup>.

Co-expression of MPGES1 and COX-2 in several tissues led to the assumption that these two enzymes may also be functionally coupled. This was demonstrated in rat peritoneal macrophages, synovial cells, gastric tissue and symptomatic atherosclerotic plaques<sup>110</sup> where co-ordinate upregulation of COX-2 and MPGES1 was followed by a major PGE<sub>2</sub> response. Also, co-localization in endothelial cells from the blood-brain barrier in response to peripheral inflammation, such as adjuvant induced arthritis, suggests a coupled system that elicits PGE<sub>2</sub> brain formation with subsequent fever and pain perception. Parallel suppression of MPGES1 and COX-2 reflects their co-induction, as dexamethasone abolishes enzyme formation and PGE<sub>2</sub> production in A549 cells, synoviocytes, macrophages and osteoblasts<sup>110,133,158</sup>.

Several experimental models of acute and chronic inflammation in rats or knockout mice as well as human studies have demonstrated the role of MPGES1 and COX-2 in arthritis. In rat adjuvant induced arthritis<sup>159,160</sup> as well as carageenan-induced paw inflammation<sup>161</sup>, induction of MPGES1 and COX-2 was found in the inflamed rat paw. Mice lacking MPGES1 develop a milder collagen induced or antibody induced arthritis compared to wildtype controls, have lower incidence and less evidence for bone destruction<sup>162,163</sup>. A similar arthritic phenotype is obtained in mice lacking cPLA2, COX-2<sup>164</sup> or EP4 receptors<sup>136</sup>, suggesting a role for the cPLA2-COX-2-MPGES1-EP4 axis in pathogenesis of at least rodent arthritis. As opposed to COX-2, mice lacking COX-1 were not protected from developing the disease and they had similar incidence and histopathology findings as wildtype COX-2 mice. Antigen-induced paw edema and pain is markedly reduced in MPGES1 knockout mice<sup>162</sup>, with associated less cellular infiltration. MPGES1 derived PGE<sub>2</sub> also plays a role in angiogenesis, since MPGES1-/- mice display less VEGF expression in the granulation tissue and reduced vessel formation.

In humans, MPGES1 and COX-2 are present in the rheumatoid arthritis synovial lining cells, mostly fibroblasts and macrophages<sup>134</sup>, with higher expression levels in active versus inactive arthritis<sup>154</sup>. Several factors present in the rheumatic joint upregulate MPGES1 expression, usually in concert with COX-2, as is the case for the classical inflammatory mediators IL-1 $\beta$ , TNF, IL-6<sup>165,166</sup>, but also for adiponectin<sup>167</sup> and epidermal growth factor<sup>168</sup>. In addition, PGE<sub>2</sub> can autoregulate its own production by increasing MPGES1 gene expression, a mechanism possibly responsible for a vicious circle of inflammation<sup>125</sup>. Parallel downregulation of COX-2 and MPGES1 by glucocorticoids might involve suppression of NF- $\kappa$ B and Egr-1 transcription and was demonstrated in a number of *in vitro* systems<sup>133,158</sup>. Both chondrocytes and synovial fibroblasts in osteoarthritis display MPGES1 and COX-2 expression that can be upregulated by inflammatory cytokines, similarly to RA<sup>169</sup>.

COX-2 and COX-1 are both expressed in synovial tissue of RA patients and in synovial cells<sup>170,171</sup>, while osteoarthritic tissue displays less COX-2 expression<sup>172</sup>. Increased expression in time was observed for COX-2 during development of arthritis in rats<sup>170,173</sup>, as well as the appearance of MPGES1 positive cells in CIA rat paws even

before clinical onset of the disease (Westman M. unpublished data), suggesting a role for this system in the early pre-clinical phase of arthritis in rats.

In all, existing data points to inducible PGE<sub>2</sub> as important contributor to rheumatoid arthritis pathogenesis, through expression of its synthesizing enzymes in the synovial tissue, high levels of PGE<sub>2</sub> in the fluid and marked reduction in disease severity in rodent models lacking an active pathway.

However, the role of PGE<sub>2</sub> in promoting inflammation is not generally accepted and has been questioned in RA by the observation that NSAIDs can increase TNF production while addition of PGE<sub>2</sub> can reverse this effect<sup>174</sup>. This, together with the fact that NSAIDs have not proven to retard disease progression, have led to the hypothesis that pathogenic mechanisms in rodents may not fully reflect the events in human RA.

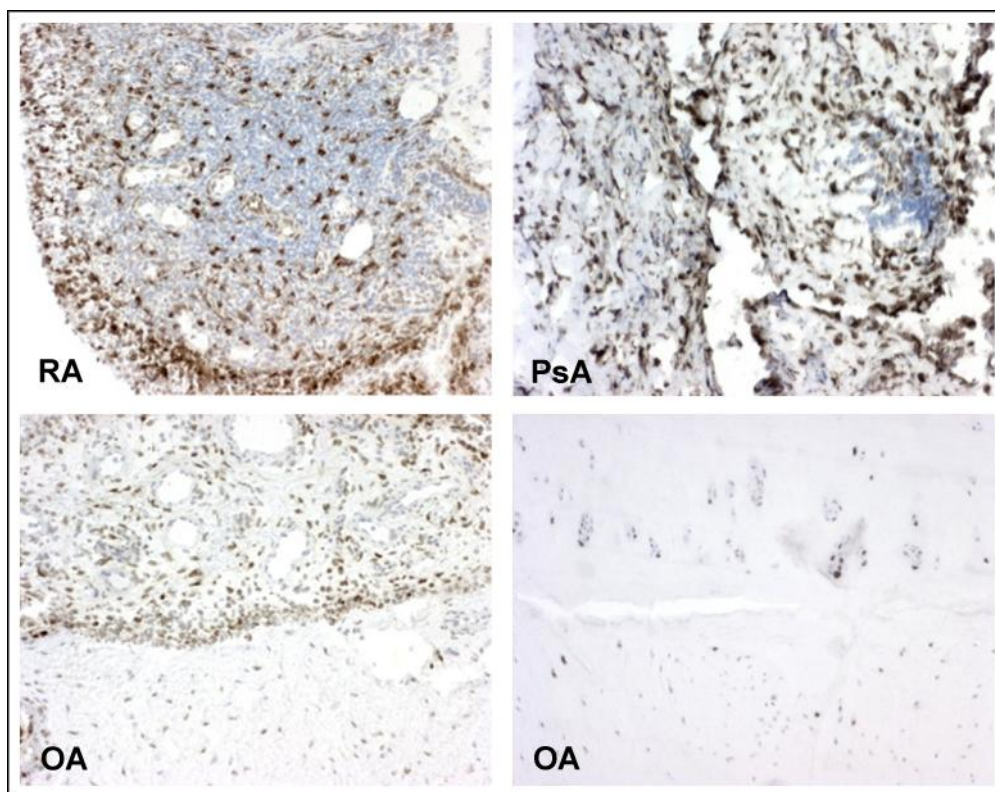


Fig.2 MPGES1 expression in rheumatic conditions associated with synovial inflammation. MPGES1 positive cells (brown) are seen in RA, PsA and OA synovial membranes and also in OA chondrocytes.

#### PGE<sub>2</sub> degradation - 15-PGDH

15-PGDH is the key enzyme in the metabolism of prostaglandins, being responsible for reducing PGs to compounds with minimal biological activity. Apart from PGs, monocyte-derived 15-PGDH can also degrade lipoxin A<sub>4</sub><sup>175</sup> and a few other eicosanoids<sup>176</sup>. It is a 29kDa protein expressed in most tissues, with high levels in lungs where most PG degradation occurs. Interestingly, there are reports showing reciprocal regulation of COX-2 and 15-PGDH, for instance IL-1 $\beta$  and TNF upregulate COX-2 while decreasing 15-PGDH expression. In addition, indomethacin, a NSAID blocking

COX-2 increases its expression, and IL-10 relieves the suppressing action of TNF and IL-1 on 15-PGDH. Furthermore, vitamin D treated neonatal monocytes increase 15-PGDH transcription while decreasing COX-2, with a net decrease in PGE<sub>2</sub> during osteoclastogenesis<sup>177</sup>. As part of the systemic inflammatory response, the transcriptional downregulation of 15-PGDH contributes to an increased PGE<sub>2</sub> level<sup>178,179</sup>.

15-PGDH has received most attention in cancer-related studies, in which low levels of the enzyme were shown to correlate with cancer progression, in line with COX-2 overexpression and high levels of PGE<sub>2</sub> promoting carcinogenesis. Reduction in 15-PGDH expression is a poor prognostic factor and is associated with an aggressive phenotype in several types of malignancies<sup>180,181</sup>. In inflammatory bowel disease- an autoimmune disorder- the inflamed mucosa displays lower levels of 15-PGDH than unaffected tissue, while *in vitro* TNF suppresses 15-PGDH transcription in colonocytes. Surprisingly few studies investigated 15-PGDH in arthritis, although the pathogenic role of PGE<sub>2</sub> in this context has long been recognized. In mouse articular chondrocytes, IL-1 and adipocyte-derived factors were found to inversely regulate MPGES1 and 15-PGDH; the constitutively expressed 15-PGDH mRNA is decreased while MPGES1 is enhanced<sup>182</sup>.

### Lipoxygenase pathway

There are several lipoxygenases catalyzing the formation of a large number of compounds with different biological activities. Pathways including 5-lipoxygenase and 15-lipoxygenase have high relevance for inflammatory disorders, as they form potent pro-inflammatory mediators largely studied in conditions such as asthma, cardiovascular disorders and arthritis. These enzymes also synthesize anti-inflammatory compounds involved in limiting the inflammation extent and restoring tissue homeostasis.

Stemming from the common precursor arachidonic acid, leukotrienes are generated after initial formation of an intermediate leukotriene (LT) A<sub>4</sub> by 5-lipoxygenase. Subsequently, LTA<sub>4</sub> can follow the pathway leading to cysteinyl leukotrienes or LTB<sub>4</sub> synthesis. LTA<sub>4</sub> hydrolase gives rise to a potent proinflammatory eicosanoid termed LTB<sub>4</sub>, while LTC<sub>4</sub> synthase forms LTC<sub>4</sub> and in subsequent steps, LTD<sub>4</sub> and LTE<sub>4</sub>, compounds generally referred to as cysteinyl leukotriens (CysLT), well known for their role in asthma pathogenesis.

15-LO acting on AA gives rise to 15-hydroxitetraenoic acid (15-HETE) and when acting in concert with 5-LO through transcellular metabolism, can form anti-inflammatory lipoxins. A new class of compounds termed eoxins is synthesized in eosinophils and mast cells through the 15-LO pathway and are believed to promote vascular permeability with edema formation<sup>183</sup>.

5-lipoxygenase and LTB<sub>4</sub>

5-LO, a key enzyme in LTB<sub>4</sub> synthesis, is mostly expressed by cells of myeloid lineage, particularly neutrophils, monocytes/ macrophages, mast cells, basophils and B cells. It is a 78kDa soluble protein with variable cell localization requiring calcium for its activity. Whether located in cytosol or nucleus, 5-LO translocates to the nuclear

membrane upon cell stimulation, while 5-LO activating protein (FLAP) transfers AA to 5-LO for efficient 5-LO activity. In monocytes, 5-LO expression is increased during migration and differentiation into macrophages.

LTB<sub>4</sub> is one of the most potent inflammatory mediators among eicosanoids, with a wide range of effects on leukocyte functions. LTB<sub>4</sub> signals through two types of receptors. BLT2 has low affinity for LTB<sub>4</sub> and is more widely expressed in human tissues; BLT1 is a high affinity receptor expressed predominantly in leukocytes, including neutrophils, monocyte/ macrophages, mast cells, T lymphocytes and mast cells<sup>184,185</sup>.

LTB<sub>4</sub> has broad functions in regulation of the immune system including neutrophil chemotaxis, migration through endothelium and activation<sup>186,187</sup>; activation of adhesion molecules expression<sup>188</sup>, activation and induction of cytokine production in monocytes<sup>189</sup> as well as activation of B cells and T cells<sup>190,191</sup>. Th1 and Th2 cells upregulate BLT1 expression on their surface while migrating out of the lymphoid organs and this receptor mediates the LTB<sub>4</sub>-induced Th cell adhesion to endothelial cells and migration to inflamed tissue<sup>192</sup>. Also, activation of dendritic cells by LTB<sub>4</sub> enhances their migration to the draining lymph nodes where they interact with antigen-specific cells. Recent reports have revealed also LTB<sub>4</sub> to be a suppressor of T regulatory cells while promoting Th17 generation, with important implications in autoimmunity<sup>193</sup>. It is essential in host defense against bacteria<sup>194</sup> and is linked to the pathogenesis of many inflammatory disorders, such as asthma, atherosclerosis and rheumatoid arthritis.

Earlier studies have documented increased levels of LTB<sub>4</sub> in synovial fluid from RA compared to degenerative arthritis<sup>195</sup> and also higher concentration in seropositive RA compared to seronegative RA<sup>196</sup>. Moreover, both receptors have been described in synovial tissue of RA patients, with higher BLT2 levels, especially in synovial lining cells<sup>197</sup>. An essential contribution of LTB<sub>4</sub> and BLT1 receptors to arthritis was demonstrated in several studies using animal models, either CIA or serum transfer model. In a study employing mice deficient in several of the LT cascade enzyme, it was shown that 5-LO and LTA<sub>4</sub> hydrolase deficient mice are markedly resistant to development of disease, but not LTC<sub>4</sub> synthase null mice, suggesting that LTB<sub>4</sub> holds a pivotal role in pathogenesis. In addition, neutrophil-derived LTB<sub>4</sub> is required for arthritis development of the disease, as transfer of these cells into 5-LO deficient mice restores the disease<sup>198</sup>. BLT1 receptor is essential in mediating LTB<sub>4</sub> effects in CIA model, since mice knocked out for this receptor are completely protected from developing arthritis<sup>199</sup>. BLT1<sup>-/-</sup> mice do not show any clinical or histopathological signs of arthritis, with complete absence of neutrophils and T cells from joints in immunized mice, suggesting an early role for LTB<sub>4</sub> and BLT1 in disease development. Moreover, not only is LTB<sub>4</sub> essential for leukocyte recruitment and activation in the joint, but also promotes their invasive and destructive behavior of fibroblast-like synoviocytes in the pannus<sup>200</sup>. As such, LTB<sub>4</sub> may play a role in the early initiating pathogenic steps but also in the progression of arthritis.

#### 15-lipoxygenase – 15-HETE and lipoxins

There are two 15-LO isoforms that differ in their enzymatic activity and tissue distribution. 15-LO-1 is constitutively expressed mostly by reticulocytes, eosinophils,

dendritic cells and airway epithelial cells<sup>201,202</sup>, whereas 15-LO-2 is present mostly in hair roots, prostate and cornea. Given the expression profile and the distinct biological functions of the two isoforms, 15-LO-1 was extensively investigated in conditions where immune responses play central roles. As 15-LO-1 participates in forming both anti-inflammatory-mediating compounds, such as lipoxins but also directly derived eicosanoids with debated biological properties, such as 15-HETE, it is not straightforward to predict how this pathway contributes to inflammatory disorders.

Expressed in a few cell types in resting conditions, 15-LO-1 can also be induced by cytokines in many different cells. Importantly, while peripheral blood monocytes do not readily express 15-LO, they are able to induce its expression through IL-4 and IL-13 stimulation while IFN $\gamma$  suppresses the induced expression<sup>203</sup>. *In vitro* differentiated dendritic cells express 15-LO and IL-13 stimulation of rabbit endothelial cells results in upregulated protein expression and generation of vasodilator mediators<sup>204</sup>.

The main compound formed by 15-LO is 15-hydroxyeicosatetraenoic acid (15-HETE) after initial synthesis of an unstable intermediate, the 15-hydroperoxyeicosatetraenoic acid. 15-HETE is a mediator with modulating effects on the immune system. There are contradictory data regarding its pro- or anti-inflammatory signaling pathways in diseases. This is in part due to a complex relationship between 15-LO products and inflammation-related signaling pathway, such as PPAR $\gamma$ , with inhibitory or activating signals depending on cell type, stimuli, disease model and other poorly understood factors.

High 15-LO expression and 15-HETE formation in airways of asthmatic individuals as well as stimulation of neutrophil chemotaxis in dog airways<sup>205</sup> suggested a role in the bronchial inflammation. In line with pro-inflammatory effects of 15-HETE, a recent study revealed its ability to stimulate NF- $\kappa$ B transcription factor and related cytokines in human monocytes<sup>206</sup>.

Lipoxins can be formed, among different pathways, by transcellular biosynthesis through coordinate actions of 5-LO and 15-LO in different cell types. Lipoxins, in particular lipoxin A<sub>4</sub>, reduce neutrophil chemotaxis and degranulation<sup>207</sup>, promote leukocyte apoptosis and their phagocytosis, thereby evoking signals that stop the inflammatory response, and contribute to resolution and restoration of tissue integrity. Lipoxin A<sub>4</sub> receptor was demonstrated to be expressed in RA biopsies, with LXA<sub>4</sub> levels increased in RA compared to OA<sup>208</sup>.

Animal models of atherosclerosis, an inflammation – related disorder, delivered conflicting results regarding the role of 15-LO. Disruption or blocking this enzyme resulted in diminished atherosclerosis<sup>209,210</sup>, with less macrophage infiltration whereas other studies showed that overexpression of 15-LO reduced atherosclerosis development with decreased size and number of vascular lesions<sup>211,212</sup>.

The relevance of 15-LO as negative regulator of inflammation has been suggested in a rabbit periodontitis model in which overexpression of 15-LO reduced inflammation and tissue damage<sup>213</sup>. In addition, mice lacking 12/15-LO (the murine homologue of 15-LO) suffered prolonged eye wound healing<sup>214</sup>. Also, macrophages from 12/15-LO knockout mice mounted an increased inflammatory response to activating stimuli and displayed abnormalities in cytokine response to bacterial products<sup>215</sup>.

In an earlier study of carrageenan-induced arthritis in dogs, intra-articular 15-HETE injection inhibited the LTB<sub>4</sub> formation and recruitment of neutrophils, along with decreased severity of arthritis<sup>216</sup>. In line with an anti-inflammatory role for 15-LO, 12/15 LO deficient mice displayed exacerbation of collagen-induced arthritis with marked destruction of the joint and increased osteoclasts number<sup>217</sup>. Further relevant to human arthritis, 15-LO-1 has been shown to be present at mRNA level in RA synovial biopsies and can be upregulated *in vitro* by IL-4 in synovial fibroblasts<sup>218</sup>.

In short, 15-LO-1 pathway and its products can display diverse actions in inflammatory conditions with many studies pointing to an anti-inflammatory effect, at least in animal models of chronic inflammation.

There is a lack of studies investigating how anti-rheumatic treatment influences locally the eicosanoid pathways with relevance to RA pathogenesis, such as PGE<sub>2</sub> and LTB<sub>4</sub> biosynthesis. Studies on the effects of anti-rheumatic treatment on 5-LO pathway have provided conflicting results. There are reports documenting inhibition of LTB<sub>4</sub> formation or 5-LO expression by glucocorticoids<sup>219</sup>, as well as opposing results<sup>220,221</sup>, with differences residing in cell type studied- peripheral blood cells, neutrophils, monocytes-, incubation time as well as concentrations used.

In addition to its effects on cytokine production and cell proliferation, methotrexate can also influence lipid derived mediators, such as cyclooxygenase derived products. However, the effects on these pathways are not entirely delineated; reports indicate decreased joint PGE<sub>2</sub> formation in rabbits receiving methotrexate, but also no effect or decrease in IL-1 induced PGE<sub>2</sub> release in cultured RA synovial fibroblasts<sup>76,222</sup>. Still, no studies were undertaken to address the local effect in the synovial compartment of methotrexate therapy on the enzymes involved in PGE<sub>2</sub> synthesis and metabolism. Hence, we felt the need to explore this pathway and the influence of common and newer anti-rheumatic drugs on enzyme expression.

Since therapy for RA may be extremely efficient for some patients, even though not curative, but not for others, it is important to investigate how these drugs target pathways known to have deleterious effects in RA, such as the eicosanoids. On one hand, are there active pathways in these patients that may explain their lack of response? On the other hand, in patients responding to treatment, can these inflammatory mediators be responsible for incomplete response or relapse, in other words, is the eicosanoid cascade targeted or not by current medication?

Through the results presented in this thesis I will try to provide some answers to these questions and a basis for possible new targets or combination therapies in RA.



## AIMS OF THE THESIS

Given the importance of lipid mediators in inflammatory reactions and their presence in the synovial fluid of rheumatoid patients, we aimed to:

- describe the distribution of enzymes responsible for their metabolism in synovial tissue
- investigate how current anti-rheumatic agents target prostaglandin and leukotriene pathways, more specifically TNF antagonists, intra-articular glucocorticoids, rituximab and methotrexate
- determine the essential structural elements in MPGES1 activity as a basis for future inhibitor studies

## RESULTS AND DISCUSSION

### **Enzymes involved in the formation of inflammatory lipid mediators are present in RA synovial tissue and are targeted by intra-articular GC therapy (Paper I, II, III and IV)**

Synovial tissue from RA patients is rich in MPGES1<sup>134</sup> enzyme while *in vitro* synoviocytes are known to upregulate expression of enzymes comprising the PGE<sub>2</sub> biosynthetic pathway when challenged with pro-inflammatory stimuli<sup>133</sup>. In our first study (**Paper I**), we extended our analysis in synovial biopsies to examine in detail the distribution of COX enzymes in relation to MPGES1 expression. By using double immunohistochemistry, we demonstrated that MPGES1 and COX-2 display similar distribution and are in most cases co-localized in intimal lining layer, unlike COX-1 positive cells that mostly lack MPGES1 expression. COX-2 expressing cells were seen among the synovial fibroblast and macrophage-like cells, but also endothelial cells in almost all patients, while only a small proportion of biopsies displayed MPGES1 positive cells in vessels. Extensive staining for COX-1 was observed both in the intimal lining and sublining layers and, similarly to COX-2 and MPGES1, was almost absent in the lymphoid infiltrate areas. As demonstrated in Paper II, MPGES1 is not expressed in B cell rich areas where most CD20+ cells and plasma cells reside, like the follicular aggregates, but in the regions surrounding these structures, likely comprising macrophages and fibroblasts.

The prostaglandin metabolizing enzyme, 15-PGDH was present in all RA synovial tissue biopsies evaluated (**Paper III**), while the extent of expression varied between samples most likely due to the degree of inflammation locally present. Not only was 15-PGDH detected in RA biopsies, but also in synovial tissue from OA and psoriatic arthritis patients, in accordance with these conditions displaying variable levels of synovial inflammation during the disease course. Most of the cells positively stained for 15-PGDH were intimal and sublining macrophages and fibroblasts, as well as endothelial cells. The fact that 15-PGDH is present in the rheumatoid milieu and shows a similar cellular distribution to MPGES1 provides an evidence the PGE<sub>2</sub> level can be adjusted locally by its synthesizing and degrading enzymes. Still, despite expression of 15-PGDH in RA tissue, PGE<sub>2</sub> is found in high concentration in the synovial fluid<sup>129</sup>, possibly reflecting an intense biosynthetic activity. Indeed, potent and numerous MPGES1 stimulatory mediators including IL-1, TNF, IL-6 are all capable of sustaining constant PGE<sub>2</sub> formation.

Leukotriene synthesis is clearly ongoing in the rheumatoid joint, since the synovial fluid is rich in LTB<sub>4</sub> and FLS<sup>200</sup>, monocytes<sup>223</sup>, neutrophils<sup>224</sup>, mast cells<sup>225</sup> and osteoclasts<sup>226</sup> are capable of producing LTB<sub>4</sub> when appropriately stimulated. Animal studies have documented the importance of 5-LO pathway in RA pathogenesis and there are clinical trials evaluating the efficacy of blocking this pathway<sup>227</sup>. We underwent a detailed analysis of 5-LO and 15-LO expression in the synovium of RA patients and employed immunofluorescence technique to phenotype the cells

responsible for enzyme synthesis (**Paper IV**). In the rheumatoid synovium, 5-LO is present in the synovial lining and in scattered cells in the sublining, with a staining pattern suggesting nuclear localization. Most of the 5-LO positive cells are resident (CD163) and migrated (CD68) macrophages; even though in low number, mast cells and neutrophils can express 5-LO while T cells and, surprisingly B cells, do not. Several studies have documented LTB<sub>4</sub> formation by B cells and it is well accepted that 5-LO is expressed in these cells. Recently, expression of 5-LO was evaluated in B cell subsets and shown to be high in immature mantle zone IgD<sup>+</sup> B cells, whereas plasma cells and germinal center B cells almost lack this enzyme<sup>228</sup>. On the other hand the majority of B cells in the synovium are memory CD27<sup>+</sup> cells and very few naïve IgD<sup>+</sup> cells<sup>22</sup>. Taken together, the lack of 5-LO expression in synovial B cells is a result of the specific B cell subsets present in the synovial tissue.

15-LO enzyme was present both in the lining synovial cells and in the sublining region, mostly expressed by cells of the monocyte lineage and fibroblasts. In addition, most vessels in the tissue displayed 15-LO expression in CD31<sup>+</sup> endothelial cells. As for 5-LO, no expression was revealed in T and B lymphocytes comprising inflammatory infiltrates. As opposed to RA, samples obtained from patients undergoing joint replacement surgery for osteoarthritis showed less pronounced expression of lipoxygenases. Indeed, chondrocytes and cartilage areas do not display enzyme expression (data not shown), whereas the few synovial membrane areas showed discrete 5-LO staining in macrophage-like cells; as a common feature to RA, 15-LO expression was detected in vessels and synovial lining cells.

Glucocorticoids are highly efficient anti-inflammatory drugs and have a special place as adjuvant medication in RA but also in other inflammatory arthropathies owing to quick relief of inflammation and related symptoms, but also to their proven bone-protective effect in low-dose oral administration and as intra-articular injection. We have studied the influence of intra-articular therapy on the eicosanoid pathway in synovial tissue (**Paper I, III, IV**) and shown that enzymes involved in formation of potent inflammatory mediators are targeted by such treatment. In particular, MPGES1, COX-2, and surprisingly COX-1, all responsible for the PGE<sub>2</sub> metabolism, as well as 5-LO, involved in formation of leukotrienes showed decreased synovial expression following local therapy with glucocorticoids. Despite most often regarded as a constitutive protein with ubiquitous expression, COX-1 can also be upregulated in response to IL-1 in synovial fibroblasts<sup>229</sup>. In this system, dexamethasone was shown to downregulate both COX-1 and COX-2 expression, which is in line with our results. Possibly, COX-1 induction is cell-specific and stimulus-dependent and in RA it is sensitive to mediators present in the inflamed tissue.

As expected for a constitutive enzyme, cytosolic PGES was not influenced by intra-articular GC. Although not achieving statistical significance, 15-PGDH showed a trend towards reduced expression (p=0.07), suggesting its dual role as regulator of catabolism for both pro-inflammatory and anti-inflammatory compounds. When it comes to 15-LO expression, albeit not significantly influenced at the group level, it was reduced in a high proportion of patients, while only 2 out of 11 showed increased staining. While 15-HETE, the main product of 15-LO-1 is regarded as a pro-inflammatory mediator, many studies claim the beneficial role for 15-LO-1 pathway in inflammation through

participation in lipoxins synthesis. As such, transgenic TNF mice lacking 15-LO showed a much more severe arthritic phenotype than those having an active 15-LO pathway with increased systemic expression of IL-6 and IL-1<sup>217</sup>. Moreover, the levels of lipoxin A<sub>4</sub> were decreased in knockout animals and correlated with high expression of pro-inflammatory cytokines and tissue damage, suggesting an important contribution of lipoxins to the modulation of inflammatory response. In this study, 15-HETE displayed anti-inflammatory properties by reducing TNF-induced IL-6 in macrophages. We have shown that in the synovial fluid, 15-LO can catalyze the formation of 15-HETE upon exogenously added arachidonic acid, albeit the amounts detected were rather small. Moreover, we could not detect any 15-HETE in freshly isolated synovial fluid, since the values were under the detection limit for the enzyme immunoassay method we used. This can also be due to the fact that readily formed 15-HETE can undergo transformation by 5-LO into LXA<sub>4</sub>, a common pathway for lipoxin biosynthesis<sup>230</sup>, since both these enzymes are present in the tissue and synovial cells. In this sense, increased expression of 15-LO after GC treatment as seen in 2 of our study patients may be related to better anti-inflammatory effects.

Earlier studies have documented the inhibitory effect of GC on leukotriene pathway, by reducing both expression of 5-LO and formation of LTB<sub>4</sub><sup>196,219,231</sup>, which is in line with our results. Knowing the role of LTB<sub>4</sub> in inducing bone loss in inflammatory conditions<sup>226</sup>, reduced LTB<sub>4</sub> levels as achieved by GC may contribute to their bone-protective effect. In addition, as LTB<sub>4</sub> is a potent chemotactic agent, another consequence of lowering its concentration is diminished cellularity in the synovial tissue. As such, reduced 5-LO expression with consequent decrease in leukotriene production in the rheumatoid milieu provides additional mechanisms by which intra-articular GC therapy exerts its beneficial effects.

Despite elevated LTB<sub>4</sub> levels in several inflammatory conditions and strong evidence from animal and *in vitro* data for a pathogenic role, clinical trials evaluating inhibitors of this pathway have been disappointing and failed to show efficacy so far<sup>227</sup>. Possibly, the assays evaluating these compounds in peripheral blood cells were not suitable to reflect the effects at the site of inflammation<sup>232</sup>. Another aspect is the heterogeneity of inflammatory diseases, with possible variation in the levels of LTB<sub>4</sub> among subgroups of patients, so that the patient population needs to be carefully selected. Lastly, RA is a disorder with complex network of mediators, where a combination therapy may prove more efficient than monotherapy.

### **Lipid mediators of the innate immune response are refractory to TNF blocking, B cell depletion or methotrexate treatment**

The development of TNF blockers and their success in RA treatment has opened new possibilities for the care of rheumatic patients and provided a proof of concept that targeting distinct pathways may result in profound therapeutic benefit. It also set the ground for investigating and understanding which processes in the joint are essential for efficient response to treatment. The efficiency of B cell depleting therapy in RA underscores the role of B cells in coordinating immune responses in the synovium and suggests that disruption of the autoreactive circuits may be central in re-establishing immune homeostasis. Methotrexate has long held a central role in RA therapy and its

ease of administration, acceptable safety profile and good efficiency in terms of protection from joint destruction<sup>233</sup> have made it the dominant DMARD and the first choice in treatment during the past couple of decades<sup>18</sup>. We chose therefore to investigate how these efficient RA therapies influence the prostaglandin E<sub>2</sub> biosynthetic pathway, known to regulate important processes in the disease pathogenesis.

We investigated the expression of MPGES1 and COX-2, the inducible enzymes of the PGE<sub>2</sub> axis in synovial biopsies obtained from two groups of patients treated with TNF blockers (**Paper I**); the first group included 8 patients receiving etanercept and the second group 10 patients with infliximab treatment. No significant influence in the expression of either enzyme could be detected for any of the two TNF blockers evaluated. TNF is an important contributor to MPGES1 and COX-2 upregulation in different cell systems<sup>158,165,234</sup>; still other more potent stimulators also promote PGE<sub>2</sub> induction along with TNF. Indeed, we and other investigators have demonstrated that IL-1 $\beta$  induces a stronger upregulation of PGE<sub>2</sub> (Fig. 3) and MPGES1 mRNA<sup>133</sup> than TNF in cultured synoviocytes; moreover, IL-1 expression may remain unchanged following treatment with TNF blockers<sup>235</sup>. Also, HMGB1 (high mobility group B1) is another candidate pro-inflammatory molecule expressed in RA tissue<sup>236</sup>, able to induce PGE<sub>2</sub><sup>237</sup> and not targeted by anti TNF therapy<sup>238</sup>. As a result, induction of PGE<sub>2</sub> producing enzymes continues despite important reduction in the synovial inflammatory load. On the other hand, therapy with TNF antagonists was shown to diminish monocyte/ macrophage population<sup>94</sup>, where much of the MPGES1 and COX-2 reside, besides fibroblasts, and to reverse NF- $\kappa$ B activation<sup>239</sup>. This could hypothetically result in lower number of cells expressing these enzymes and reduced levels of pro-inflammatory mediators that could induce them. However, our data suggest that the level of enzyme expression is not a simple mathematical compilation of numbers of cells and mediators, but the result of a complex network of cell interactions and regulatory feedbacks.

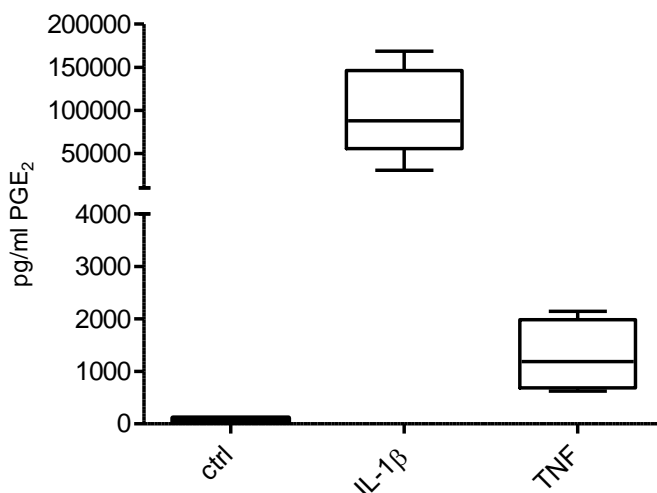


Fig. 3 Formation of PGE<sub>2</sub> in RA cultured fibroblast-like synoviocytes treated with IL-1 $\beta$  or TNF for 24h.

In order to avoid confounding effects in the synovial analysis, patients treated with rituximab (**Paper II**) did not receive prednisolon pre-treatment along with the

infusions, as is the standard administration procedure to minimize risks for allergic reactions. Biopsies were prelevated from 24 patients before first infusion as well as at two consecutive timepoints, 4 and 16 weeks later. Patients enrolled in this study had active RA, defined as  $\geq 4$  swollen and tender joints in the 28 joints assessed and at least one of the following criteria: erythrocyte sedimentation rate  $\geq 28$ mm/h, serum C-reactive protein  $\geq 15$ mg/ liter, or morning stiffness  $\geq 45$ min. In addition, all patients were positive for either rheumatoid factor or ACPA and had active arthritis in at least one joint accessible for arthroscopy. Investigation of MPGES1 and COX enzymes revealed that their expression remains essentially unaffected by this therapy and no association pattern can be seen with clinical course or depletion of CD22 positive B cells in the synovial tissue (data not shown). We next analyzed cytokines known to induce these enzymes in the rheumatoid compartment and demonstrated that indeed rituximab, despite depleting macrophage and T cell populations<sup>100</sup> in the tissue, has limited influence on IL-1 $\beta$  and IL-6 expression.

An important implication of an earlier finding that antibody production depends on COX-2 expression in peripheral blood B cells<sup>240</sup> is that autoantibody formation as seen in rheumatoid arthritis may be altered by interventions targeting this pathway. However, we were not able to demonstrate any MPGES1 expression in either CD20+ B cells or plasma cells in the synovium so it is unlikely that these cells form PGE<sub>2</sub> even though some might express COX-2. We know that B cells in the peripheral blood may differ substantially in behavior from synovial fluid B cells (see later *in vitro* results), and possibly also from the repertoire present in the rheumatoid synovium<sup>22</sup>, although detailed phenotypic comparative analysis is lacking. It seems that B cells in synovial tissue are not a source of PGE<sub>2</sub>, but may however respond to it through EP receptors present on their surface<sup>241</sup>. In addition, persistent PGE<sub>2</sub> during rituximab treatment may herald relapse by supporting viability of replicating B cells<sup>242</sup> that were not completely removed from the tissue, thus promoting B cell expansion in the synovium. PGE<sub>2</sub> is a complex stimulus, given the B cell expression of four EP receptors, so, while trying to understand the effects of PGE<sub>2</sub> on synovial B cells, it is important to keep in mind that: 1) the sensitivity to PGE<sub>2</sub> depends on B cell developmental state<sup>243</sup>; and 2) each EP receptor subtype mediates unique functional responses. It was recently shown that PGE<sub>2</sub> promotes survival pathways in dividing B lymphoblasts through upregulation of EP2 receptor<sup>242</sup>, while in another study PGE<sub>2</sub> suppressed B cell proliferation via EP4 receptors<sup>121</sup>. As such, the net result of remnant PGE<sub>2</sub> in the synovial tissue after rituximab therapy remains to be further evaluated; however, persistence of other cytokines as IL-1 $\beta$  and IL-6 suggests an active ongoing inflammation.

In the methotrexate study group (**Paper III**), we extended our analysis to include enzymes involved both in synthesis and degradation of PGE<sub>2</sub>. As such, we studied the expression of MPGES1, COX-1, COX-2 and 15-PGDH in a group of 13 early RA patients naïve for methotrexate treatment and newly diagnosed. After a median of 8 weeks following therapy start, synovial biopsies showed no change in enzyme expression either in the whole group or when analyzing subsets of patients stratified on response to treatment or autoantibody positivity. However, the small size of our study group, with few patients in each analyzed subgroup precludes a definite conclusion in the general RA population, but rather gives a hint that at least in some patients this

pathway remains functional. Validation in a larger cohort or evaluation of pre-defined subsets of patients may reveal effects specific for distinct pathogenic groups.

Methotrexate was shown to inhibit the expression of IL-1 $\beta$  in RA synovial tissue<sup>244</sup> and that of TNF in responder patients<sup>245</sup> together with reduction in macrophages, T cells and plasma cells four months after therapy start. The decrease in adhesion molecules<sup>244</sup> is thought to account for the reduced cellularity. Alternatively, the induced adenosine may inhibit leukocyte accumulation<sup>78</sup>. We demonstrated in this study that in early RA PGE<sub>2</sub> pathway remains unaffected after the first 8 weeks of treatment. Since usually a trial of three months is allowed for evaluation of efficacy and additional improvement in disease activity can be achieved after this period<sup>246</sup>, later effects cannot be excluded. In short, we can conclude that targeting PGE<sub>2</sub> is not one of the mechanisms responsible for early methotrexate efficiency. Despite significant reduction in RANKL expression in these patients<sup>247</sup>, some show ongoing bone destruction to which persistent PGE<sub>2</sub> may contribute.

### **Distinct effects of *in vitro* anti-rheumatic treatment on RA synovial cells (Paper I, II and III)**

Synovial fluid mononuclear cells (SFMC) were evaluated *in vitro* for the expression of PGE<sub>2</sub> pathway enzymes in resting and stimulated conditions (**Paper I**). LPS is a classical and potent inducer of these enzymes, useful for studying effects of various treatments in activated monocytes that are sensitive to its stimulation. In addition, it mimics TLR ligands present in the rheumatoid joint. Both dexamethasone and the TNF antagonists tested – infliximab and etanercept- decreased the expression of MPGES1 and COX-2 in CD14+ monocytes, while not influencing COX-1 expression. Accordingly, PGE<sub>2</sub> accumulated in the culture supernatants as well as the amount formed after incubation of cultured cells with exogenous arachidonic acid mimicked the dynamics of enzyme expression – strong induction by LPS followed by downregulation by dexamethasone and anti-TNF agents. Glucocorticoids are strong inhibitors of the inducible enzymes MPGES1 and COX-2, without affecting constitutive COX-1, as demonstrated by studies in numerous *in vitro* systems. Infliximab and etanercept act by blocking TNF actions and may well result in interference with LPS-induced PGE<sub>2</sub> system, since LPS stimulates the release of both TNF and IL-1 $\beta$ , either of which can induce MPGES1 and COX-2. Pro-inflammatory cytokines can exert distinct and specific effects on different cell types, depending also on the density and type of surface receptors. It was previously reported that anti-TNF treatment of mouse macrophages attenuates COX-2 expression in response to NF- $\kappa$ B activation<sup>248</sup>, while in other investigators reported that anti-TNF antibodies failed to block LPS-induced COX-2<sup>249</sup>. Our results suggest that in LPS-stimulated SFMC, the COX-2-MPGES1- PGE<sub>2</sub> axis is TNF dependent.

*Staphylococcus aureus* Cowan strain I (SAC) and pokeweed mitogen (PWM) are polyclonal B cell activators that induce B cell proliferation and activation; while SAC stimulates B cells independently of T cells, PWM is T cell-dependent. We used this powerful combination of stimuli to mimic the complex network of activating mediators in the rheumatic milieu and to assess the ability of B cells to express the enzymes of

interest while fully activated (**Paper II**). As demonstrated by flow cytometry analysis of CD19+ gated cells, resting B cells did not display MPGES1 or COX-2 enzymes either in periphery or in the synovial fluid, but once activated, synovial fluid B cells upregulated the expression of both enzymes to a higher level than peripheral blood B cells. This is in accordance with previous studies<sup>122,240</sup> documenting COX-2 and PGE<sub>2</sub> induction in stimulated peripheral B cells. The difference in the level of expression attained by B cells originating from synovial fluid versus B cells from peripheral blood may be explained by specific subsets or activation states in the two compartments. A recent investigation reported MPGES1 expression in replicating cells<sup>242</sup>, as is the case in our experimental setting where the stimuli we used determine B cell proliferation. However, in the RA tissue there is little B cell proliferation<sup>22</sup> and also rather few naïve B cells; there is also evidence that distinct B cell subsets may be activated by different factors<sup>250</sup>; therefore, the lack of MPGES1 expression in tissue B cells as detected by immunohistochemistry possibly reflects the difference in B cell subsets between tissue and fluid / blood but also the fact that our *in vitro* system may not faithfully reproduce the rheumatic environment.

Fibroblasts-like synoviocytes were cultured *in vitro* and stimulated with IL-1 $\beta$  or TNF for 24h and 48h in order to test the best conditions for MPGES1 and COX-2 induction (**Paper III**). In subsequent experiments IL-1 $\beta$  stimulation was applied for 48h as this provided the strongest PGE<sub>2</sub> formation. *In vitro* treatment with methotrexate was added as 10 $\mu$ M and 250 $\mu$ M, as the usual concentration attained in blood upon low-dose weekly therapy is in the 100  $\mu$ M range<sup>251</sup>. Both on a protein level, as assessed by MPGES1 and COX-2 western blotting, and on a functional level, reflected by formation of PGE<sub>2</sub> and other eicosanoids of the cyclooxygenase pathway, methotrexate exerted no detectable effects. We observed stable protein levels and non-significant changes in prostaglandin formation in treated cells compared to controls.

There are inconclusive studies regarding methotrexate effects on cytokine and lipid derivatives production *in vitro*. In synovial fibroblasts, methotrexate had no effect on IL-1 stimulated cytokine and PGE<sub>2</sub> production<sup>222,252</sup>, while other study claimed reduced PGE<sub>2</sub> level<sup>253</sup> although in this latter investigation IL-1 was only added towards the end of the incubation period. It is also suggested that methotrexate inhibits TNF-induced NF- $\kappa$ B activation<sup>254</sup>. However, IL-1 and TNF, despite having sometimes redundant functions, differ in signaling pathways<sup>255</sup>. Taken together, the IL-1 $\beta$ - induced PGE<sub>2</sub> pathway in fibroblasts is resistant to methotrexate treatment, a feature in accordance with results obtained in *ex vivo* analysis of the same enzymes.

MPGES1 and related enzymes persist in synovial tissue, particularly in fibroblasts despite anti-rheumatic treatment (see Paper II). These cells are capable to invade cartilage and start degradation processes in the absence of other effector molecules and also migrate from inflamed to non-affected joints<sup>256</sup>. It is well-known that PGE<sub>2</sub> holds a central role in the aggressive nature of cancer cells<sup>257</sup>. Since RA pannus is sometimes regarded as a locally invasive tumor<sup>6</sup>, it is tempting to speculate that PGE<sub>2</sub> could promote a similar character in synovial fibroblast-like cells and account for their invasive nature. This remains however a hypothesis that needs to be tested.



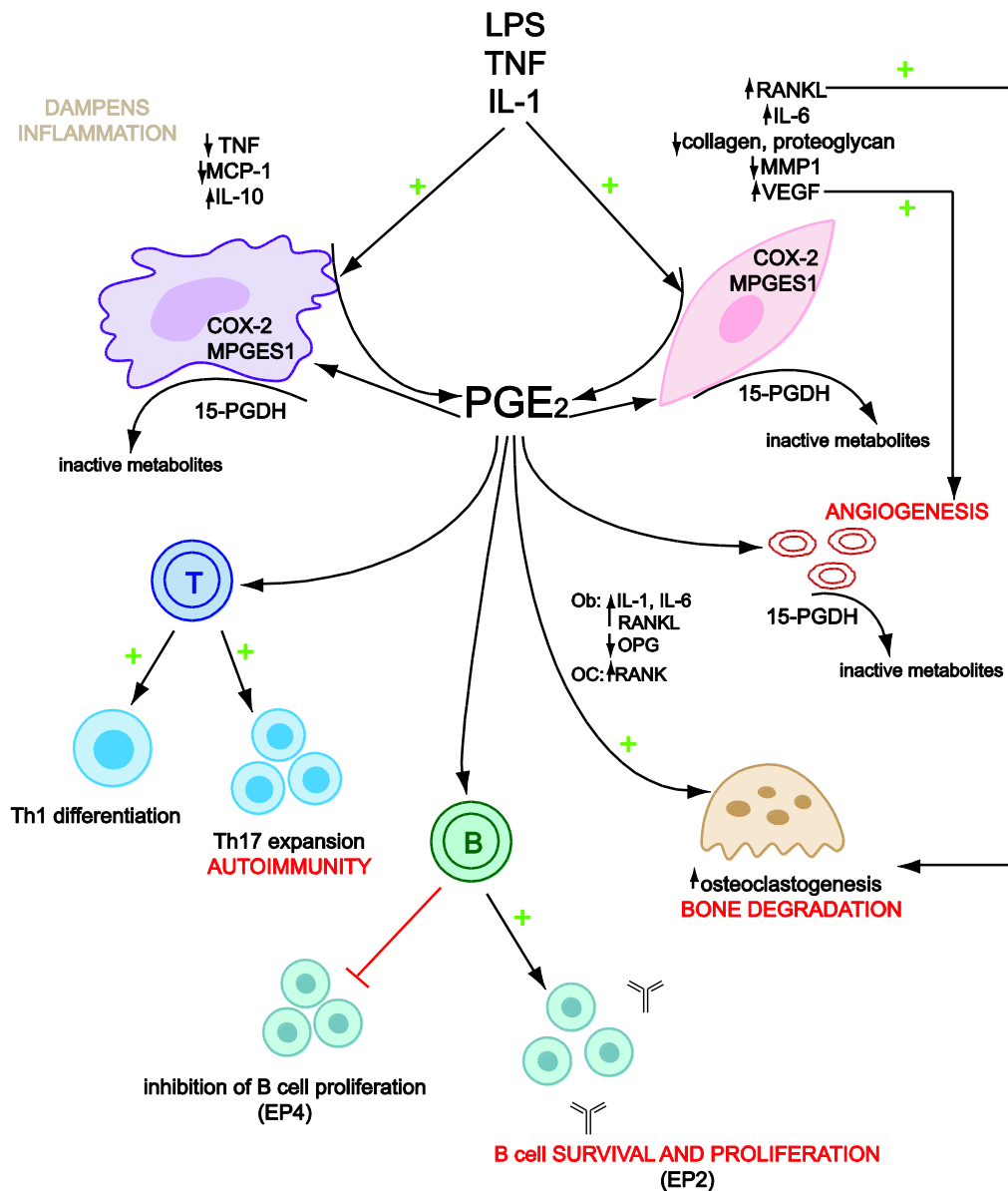


Fig. 4 Cellular expression of PGE<sub>2</sub> pathway enzymes in RA synovial tissue and the contribution of cells involved in this pathway to disease pathogenesis. PGE<sub>2</sub>, mainly produced in synovial macrophages and fibroblasts by inflammation-induced COX-2 and MPGES1, promotes formation of new vessels (through increased VEGF expression), bone destruction (through reduced OPG and enhanced RANKL expression in fibroblasts and osteoblasts) and perpetuation of autoimmune reactions (through B cell survival and proliferation and Th17 expansion). At the same time, PGE<sub>2</sub> may also dampen inflammation by reducing TNF and increasing IL-10 in macrophages. Macrophages and fibroblasts, together with endothelial cells, are also the cells capable of PGE<sub>2</sub> removal by inactivation.

### Structural determination of human MPGES1 (Paper V)

Large well-ordered two-dimensional crystals of purified human MPGES1 together with glutathione were grown in the presence of phospholipids. We determined the

structure by cryo electron microscopy to a resolution of 3.5 Å and showed that the enzyme displays four transmembrane  $\alpha$ -helices and forms a trimer in the membrane, similar to other MAPEG proteins<sup>258</sup>. The active site where the substrate PGH<sub>2</sub> gains access is found at the interfaces between two monomers and is stabilized by glutathione through specific aminoacid interactions. Comparison with LTC<sub>4</sub> synthase<sup>259,260</sup> suggests that there is a conformational change with an open MPGES1 structure allowing for substrate access. In addition, key aminoacids specific for the human enzyme, not present in rat or mouse proteins, participate in the interactions at the active site and may explain the lack of effect on rodent enzyme of inhibitors developed towards human MPGES1<sup>261</sup>.

The data presented here, showing limited effects on PGE<sub>2</sub> axis of traditional and newer therapies that are highly efficient in a large proportion of patients, suggests that pathways with inflammatory and destructive potential remain unaffected and may contribute to subclinical inflammation even in non-symptomatic joints<sup>262,263</sup>. It is already well-established that patients in remission present asymptomatic inflammation revealed by imaging techniques<sup>264</sup> which is responsible for progressive joint destruction. When therapy is withdrawn, such apparent silent pathways may turn on interactive immune cell communications via EP receptors present on most of these cells and induce inflammatory reactions all over again.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

During the past years, there has been an explosion of new drugs targeting specific pathways and events in the pathogenesis of RA, many of them with great impact on the disease course and quality of life for the rheumatic patient. Still, despite this tremendous improvement in therapy efficiency, there are still a high proportion of patients not adequately responding to treatment and, above all, drug-free remission has not been achieved, implying that the dysregulated inflammatory reaction is sustained by ongoing mechanisms.

The results presented in this thesis suggest that therapy with methotrexate, TNF blockers or rituximab may leave an important inflammatory pathway, the COX-2 – MPGES1 –PGE<sub>2</sub> active in the rheumatoid synovial tissue. We have demonstrated that in newly diagnosed RA, starting of therapy with methotrexate does not abrogate PGE<sub>2</sub> biosynthetic pathway in synovial tissue after 8 weeks, a result supported also by *in vitro* data. In patients treated with infliximab or etanercept, 8-10 weeks following treatment we cannot confirm any effect on the PGE<sub>2</sub> pathway. Despite *in vitro* data showing significant downregulation of this pathway, the results obtained in biopsies *ex vivo* support the idea that in order to assess the actual effect of a drug, it is important to evaluate the events at the site of inflammation. Similarly, in the cohort of patients starting rituximab treatment, biopsies obtained at 4 weeks and 16 weeks fail to provide evidence for any effect on the PGE<sub>2</sub> pathway; in addition, inflammatory mediators known to induce the PGE<sub>2</sub> biosynthetic enzymes were also not affected by this treatment. Hence, a therapeutic approach combining MPGES1 inhibitors with anti-rheumatic drugs may provide benefit in controlling synovial inflammation. And lastly, efficient anti-inflammatory glucocorticoids deplete enzymes responsible for both PGE<sub>2</sub>

and leukotriene formation in RA synovial tissue, suggesting an additional mechanism that may explain its potent effects.

It is noteworthy to mention in this context the widespread use of NSAIDs, well appreciated for relieving inflammation, pain and fever. Knowing the complexity of the eicosanoid pathway and the inter-relation between branches one can argue that blocking the whole COX pathway is a rather broad action. In this sense, reduced prostacyclin levels account for the cardiovascular side effects of these drugs, while reducing prostaglandin D<sub>2</sub> and subsequently its degradation product, 15-deoxy-delta-12-14-prostaglandin J<sub>2</sub>, may impair resolution of inflammatory processes. This suggests that drugs targeting MPGES1, the inducible enzyme in inflammatory conditions, may offer additional benefits not only by taking away inducible PGE<sub>2</sub>, but also by preserving or diverting the eicosanoid metabolism towards other bioactive lipids with pro-resolving properties.

As it is usually the case, immunomodulating molecules cannot simply be judged as black or white, and it would be wrong to say that PGE<sub>2</sub> is entirely “evil”, even in an inflammatory context. It is an important contributor to inflammation, to homing of cells at the site of inflammation while at the same time preparing the ground for resolution, by inducing lipoxin formation<sup>265</sup>. Moreover, COX inhibitors were shown to actually prolong inflammation when administered in the restoration phase<sup>266,267</sup>. In fact, only recently a very intriguing study revealed that COX-2 is involved in both the inflammatory and resolution phases in collagen induced arthritis<sup>266</sup>. By depleting COX-2-derived PGE<sub>2</sub> in the resolution phase using COX-2 inhibitors, lipoxin A<sub>4</sub> formation was blunted and tissue restoration was impaired. Interestingly, MPGES1 was not expressed during resolution in this model, so the PGE<sub>2</sub> necessary to restore tissue integrity is not dependent on MPGES1. Therefore, an attractive idea is that more targeted therapy as provided by inhibiting MPGES1 instead of COX could block inflammatory pathways while at the same time re-establishing tissue homeostasis.

PGE<sub>2</sub> may also have protective effects in wound<sup>268</sup> and fracture<sup>269</sup> healing and important functions in cardiac remodeling following infarction, as demonstrated in mice lacking MPGES1<sup>270</sup>. It will therefore be an important issue and a challenge to keep the balance between deleterious and protective PGE<sub>2</sub> effects while inhibiting MPGES1.

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