SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3276.

KÁLMÁN ESZTER GERTRÚD

Elméleti és klinikai immunológia és reumatológia

című program

Programvezető: Dr. Mócsai Attila, egyetemi tanár

Témavezető: Dr. Németh Tamás, egyetemi docens

The role of the Syk tyrosine-kinase in immune complex-mediated autoimmune inflammation

PhD thesis

Eszter Gertrúd Kálmán

Semmelweis University Doctoral School

Molecular Medicine Division

Supervisor: Tamás Németh, MD, PhD

Official reviewers: Judit Tőke, MD, PhD
Tünde Minier, MD, PhD

Head of the Complex Examination Committee:

Edit Buzás, MD, D.Sc.

Members of the Complex Examination Committee:

Attila Balog, MD, D.Sc.
Norbert Gyöngyösi, PhD
Barbara Molnár-Érsek, PhD

Budapest
2025

Table of Contents

List of Abbreviations

ACPA – Anti-citrullinated protein antibody

ADAP – Adhesion and degranulation promoting adapter protein

ADCC – Antibody-dependent cellular toxicity

anti-CVII– anti Type-VII Collagen antibody

APC – Allophycocyanin

APRIL – A proliferation-inducing ligand

BAFF – B cell activating factor

BCR – B cell receptor

Ca – Calcium

CARD9-Bcl10-MALT1 complex – caspase-recruitment domain 9–B cell lymphoma–10 Mucosa-associated lymphoid tissue lymphoma translocation protein 1 complex

CCL3 – Chemokine (C-C motif) ligand 3

CLL – Chronic lymphoid leukemia

CVII – Type-VII Collagen

CXCL – Chemokine (C-X-C motif) ligand

CXCR – Chemokine (C-X-C motif) receptor

DAG – Diacylglycerol

DAP12 – DNAX-activating protein of 12 kDa

DMARD – Disease-modifying antirheumatic drug

ECM – Extracellular matrix

EBA – Epidermolysis bullosa acquisita

ERK – Extracellular signal-regulated kinase

FAK – Focal adhesion kinase

FcR – Fc receptor

FcγR – Fcγ receptor

FCS – Fetal cow serum

Fbg – Fibrinogen

FITC – Fluorescein isothiocyanate

FLS – Synovial fibroblast

G-CSF – Granulocyte colony-stimulating factor

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GMP – Granulo-monocytic progenitor

GPCR – G protein-coupled receptor

GPVI – Glycoprotein VI

G6PI – Glucose-6-phosphate isomerase

HLA – Human leukocyte antigen

HLA-DRB*01 – Major Histocompatibility Complex, Class II, DR Beta 1

IC – Immune complex

IFN-γ – Interferon-γ

IgA – Immunglobulin A

IgE – Immunglobulin E

IgG – Immunglobulin G

IgM – Immunglobulin M

IL-1 – Interleukin-1

IL-1β – Interleukin-1ß

IL-6 – Interleukin-6

IL-8 – Interleukin-8

IL-17 – Interleukin-17

ITAM – Immunoreceptor tyrosine-based activation motif

ITIM – Immunoreceptor tyrosine-based inhibitory motif

JAK – Janus kinase

JNK – Jun N-terminal kinase

LAD – Leukocyte adhesion deficiency

LAT – Linker for Activation of T cells

LFA-1 – Lymphocyte function-associated antigen 1 (CD11a)

LTB_4 – Leukotriene B_4

Mac-1 – Macrophage-1 antigen (CD11b)

MAPK – Mitogen-activated protein kinase

Mg – Magnesium

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinase

MPO – Myeloperoxidase

NaCl – Sodium chloride

NE – Neutrophil elastase

NET – Neutrophil extracellular trap

NFAT – Nuclear Factor of Activated T cell

NF-κB – Nuclear factor κB

NLRP – Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing

NLRP3 – NLR family pyrin domain containing 3

NO – Nitric oxide

NOD mice – Non obese diabetic mice

PAD – Peptidyl Arginine Deiminase

PBS – Phosphate-buffered saline

PE – Phycoerythrin

PerCP – Peridinin-Chlorophyll-Protein

PI3K – Phosphoinositide 3-kinase

PKC – Protein kinase C

PLCγ – Phospholipase Cγ

PMA – Phorbol 12-myristate 13-acetate

PR3 – Proteinase 3

PSGL1 – P-selectin glycoprotein ligand-1

PTPN22 – Protein tyrosine phosphatase non-receptor type 22

PYK2 – Protein-tyrosine kinase 2

RA – Rheumatoid arthritis

RANK – Receptor activator of nuclear factor-κB

RANKL – Receptor activator of the nuclear factor-κB ligand

Rap1 – Ras-related protein 1

RASGRP – Ras guanyl nucleotide releasing protein

RF – Rheumatoid factor

RHO family – RAS homolog gene family

ROS – Reactive oxygen species

SEM – Standard error of the mean

SLE – Systemic lupus erythematosus

SLP76 – SH2 domain-containing leukocyte protein of 76 kDa

Syk – Spleen tyrosine kinase

$Syk^{\Delta PMN}$ mice – Animals with neutrophil-specific Syk deletion

TCR – T cell receptor

Tec – Tec Protein Tyrosine Kinase

Th1 – Helper T cell, type 1

Th2 – Helper T cell, type 2

TNF-α – Tumor necrosis-factor-α

Vav – Vav guanine nucleotide exchange factor

1. Introduction

1.1. Autoimmune diseases

Autoimmune diseases affect around 5 % of the population and the patients are mostly women (1). Autoimmune disorders can be triggered by both genetic and environmental factors. Genetic risk factors can be gene mutations, which can modify the severity of the autoimmune disease, or gene polymorphisms. There are some HLA alleles, which are associated with autoimmune disorders, like rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) (2-4). Various epigenetic factors may also contribute to the autoimmune processes, such as DNA methylation, histone modifications or non-coding RNAs (5). Most autoimmune disorders can be induced by environmental factors, like smoking or inorganic dust exposure, drugs or infectious microbial agents (2, 4, 6). As previously mentioned, women patients have a higher susceptibility to autoimmune disorders, which may be due to hormonal factors (1, 2). In the development and maintenance of an autoimmune disease, effector cells (like lymphocytes, macrophages, neutrophils, etc.) and their functions play an important role. The abnormal recognition of self-antigens by adaptive immune cells can trigger autoimmune processes, while during an inflammatory cascade, chemokines can attract innate immune cells (such as neutrophils or monocytes) from the circulation (7, 8).

Under normal conditions, pathogens can be eliminated from the body during anti-pathogen immune responses, but autoantigens cannot be removed, and the persistent presence contributes to the development and maintenance of chronic inflammatory processes (2). Autoimmune disorders can be divided into organ-specific (like epidermolysis bullosa acquisita (EBA)) or systemic disorders (like rheumatoid arthritis, systemic lupus erythematosus or systemic sclerosis, etc.) (9-13). Our experiments are prominently conducted in a mouse model of arthritis; therefore, we will focus on rheumatoid arthritis.

1.2. Rheumatoid arthritis

1.2.1. Structure of a normal and inflamed joint

Rheumatoid arthritis (RA) is a systemic and chronic autoimmune disease (14, 15). The global prevalence of RA is approximately 1 % (15). Under healthy conditions, a

peripheral diarthrodial joint is composed of articular cartilage and bones, which enclose the synovial cavity. Inside the synovial cavity we can find the synovial fluid, that is produced by the synovial membrane (Figure 1) (16).

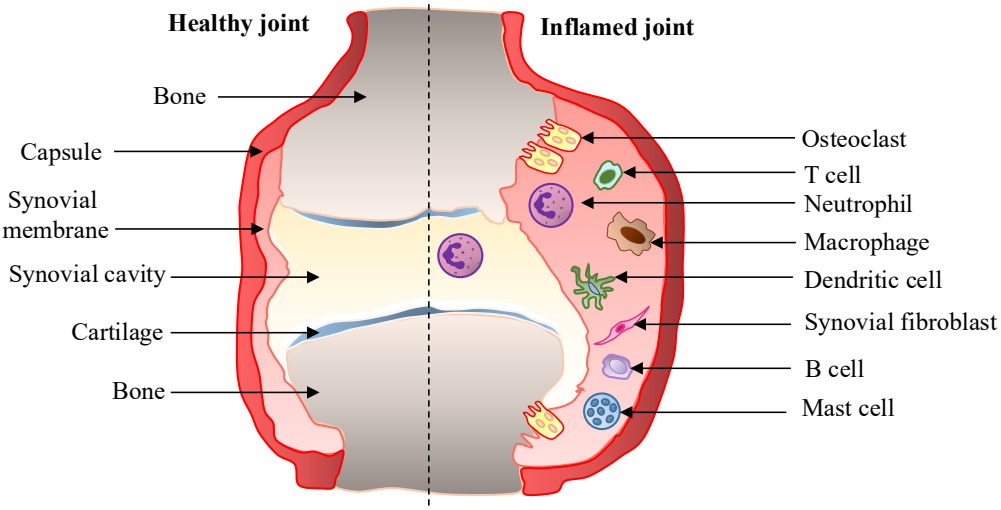

Figure 1: The healthy and the inflammatory milieu in a joint

A healthy joint is made up of bones with smooth cartilage surfaces, connected by a joint capsule. Inside the capsule lies the synovial membrane (a thin layer of cells), which is responsible for providing nutrients and enclosing the synovial cavity. The joint cavity contains synovial fluid, which is essential for lubricating the joint and maintaining its internal homeostasis. Many cell types (like osteoclasts, T and B cells, neutrophils, macrophages, dendritic cells, synovial fibroblasts and mast cells) are involved in the pathogenesis of RA. Under inflammatory conditions, they can contribute to cartilage damage and bone erosions, synovial thickening and the increase of the synovial fluid (own figure based on reference (16)).

The synovial membrane contains only a few layers of cells and it has an essential role, for example in the diffusion of nutrients. Under inflammatory conditions, this synovial membrane becomes thick and the level of the synovial fluid increase in the synovial cavity (15-17). Increased numbers of immune cells (like neutrophils, synovial fibroblasts, macrophages, osteoclasts) can be detected in an inflamed joint, with high levels of inflammatory cytokines and chemokines (e.g. TNF-α, IL-6, CXCL2, CCL3) (16, 18-21). The clinical manifestations in most patients start with pain and swelling of a few joints especially on the hands and feet. Later, RA develops into symmetric polyarthritis, which can also affect larger joints like the shoulders, the elbows, the ankles or the knees. Common features include morning joint stiffness (that last approximately half an hour or more), fatigue, weakness, weight loss or low-grade fever (Figure 2) (15).

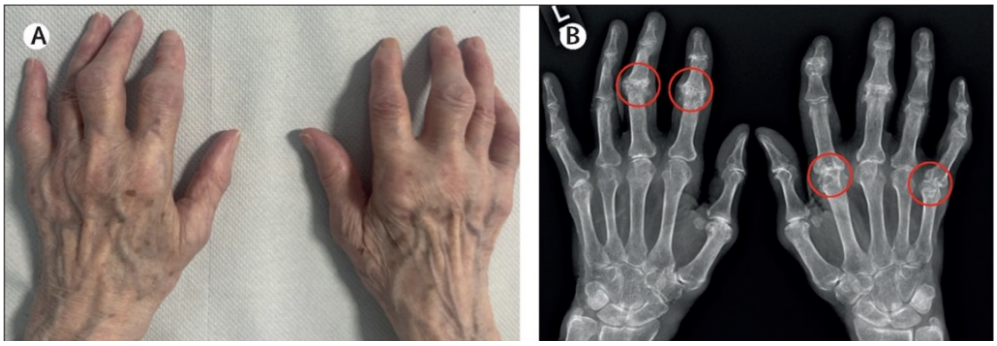

Figure 2: Long-standing rheumatoid arthritis

(a) Long-standing rheumatoid arthritis with polyarticular synovitis and joint deformities.
(b) The X-ray shows joint destruction with bone erosions and cartilage loss in metacarpophalangeal and proximal interphalangeal joint. Red circles show severe joint destructions (15).

Extraarticular manifestations can increase the mortality and morbidity of the disorder. The cardiovascular, pulmonary, neurological and renal involvements are the most common extraarticular manifestations during RA (22-27).

1.2.2. Therapeutic options

The treatment should be started with the administration of conventional synthetic disease-modifying antirheumatic drugs, like methotrexate with short-term use of glucocorticoids (28-31). If there are contraindications for methotrexate, leflunomide or sulfasalazine can be combined with glucocorticoids. If the symptoms are improved by 3 months and the remission is achieved by 6 months, the treatment can be continued (with dose reduction in sustained remission) (31). As long as the symptoms do not improve and poor prognostic factors are absent, the change to or the adding of a second conventional synthetic disease-modifying antirheumatic drug can be effective (31). If some poor prognostic factors (like high titers of Rheumatoid factor (RF) or Anti-citrullinated protein antibody (ACPA)) are present, biological disease-modifying antirheumatic drugs (like TNF-inhibitors, tocilizumab, etc.) or Janus kinase (JAK) inhibitors (like baricitinib, tofacitinib or upadacitinib) can be used after risk assessment (29-39). If the symptoms are improved by 3 months and the patient achieved the remission by 6 months, the treatment can be continued (with dose reduction in sustained remission). Assuming that the symptoms do not get better, the change to other biological disease-modifying antirheumatic drugs or JAK-inhibitors are proposed (31). In a significant proportion of

patients (sustained) remission is not achievable, so there is a need to find new drug targets and to develop novel targeted therapies as well.

1.2.3. Genetic and environmental risk factors of rheumatoid arthritis

In the development of rheumatoid arthritis, both genetic and environmental factors take part. The risk factors can be genetic disorders (like HLA shared epitope alleles, epigenetics), hormonal changes (like in postmenopause or breastfeeding or due to hormone-replacement therapies), oral health (like chronic periodontitis), respiratory exposure (like smoking) or intestinal health issues (like western diet) (16, 40-42). Epigenetic factors also contribute to the pathogenesis, like DNA methylation or histone acetylation, while genetical risk factors are principal influencers of the development of RA (40, 41). It has been described, that mutations in the Human Leukocyte Antigen (HLA) genes, which encode MHC molecules, play essential roles in the pathogenesis of RA. HLA-DRB1 is a high-risk allele, which can increase the development of early disease, bone erosions and anti-citrullinated protein antibodies during RA (40, 41, 43). In RA, citrullination can be mediated by Peptidyl Arginine Deiminase (PAD) enzymes. Citrullinated proteins can be recognized as autoantigens, which can lead to the appearance of ACPAs (41). There are also so-called non-HLA associated genetic factors, such as the Protein tyrosine phosphatase, non-receptor 22 (PTPN22) gene modification, which can trigger enhanced immune responses, for example through protein citrullination (44). Besides genetic risk factors, hormonal changes are also important. RA is more prevalent in women than in men, which can be attributed to the effects of oestrogen. It is important to mention that the postpartum period can increase the risk of RA (41, 42).

Oral health issues are also common risk factors in the pathogenesis of RA. Some oral microbes like *Aggregatibacter actinomycetemcomitans* or *Porphyromonas gingivalis* can stimulate ACPA production or some species of *Streptococcaceae* can contribute to oral dysbiosis (41).

1.2.4. The pathogenesis of RA

The pathophysiology of rheumatoid arthritis is heterogeneous. Immune complex formation can contribute to the development of joint inflammation and complement activation by anti-citrullinated protein antibodies that bind to citrulline-containing antigens (14). Under inflammatory conditions, the immune complex accumulation

corresponds to a form of type III hypersensitivity reaction, according to the Gell-Coombs classification (45). ACPAs can interact citrullinated antigens of many self-proteins, (such as fibronectin, fibrinogen or type II collagen) and the presence of ACPAs can be detected up to 10 years before the diagnosis. ACPAs have an important role in activating macrophages and osteoclasts. If they interact with vimentin, they can promote bone loss (15, 16, 41). During RA, it has been shown that Peptidyl Arginine Deiminase-4 (PAD4), which can be released during neutrophil NETosis can citrullinate certain structural proteins (like vimentin), which may trigger the production of ACPAs (46). Furthermore, ACPAs are capable of interacting with rheumatoid factor containing immune complexes, thereby enhancing and sustaining the inflammatory state (16). RF is an antibody, which is produce against the Fc part of immunglobulins and it can have elevated levels in several autoimmune diseases (e.g. in RA or in Sjögren's syndrome) (15, 16, 41). In rheumatoid arthritis, three isotype rheumatoid factor were identified (IgM, IgA and IgG), which were typically found in 52% of RA patients (47). RF has a role in macrophage activation, and the inflammatory cytokine release can be also increased (15, 16, 41). As we previously mentioned, several cell types are involved in the pathogenesis of rheumatoid arthritis, which will be discussed in the next chapter.

1.2.5. The role of immune cells in the pathophysiology of RA

Under inflammatory conditions, the number of infiltrated immune cells are increased. During the disease, the normal synovium is thickened and the inflamed membrane is infiltrated by immune cells (16, 19, 48). In the pathogenesis of RA, many cell types are involved, like lymphocytes, neutrophils, macrophages or synovial fibroblasts. Here, we provide a more detailed overview of the role of synovial fibroblasts and neutrophils.

Synovial fibroblasts are located in the synovium in the lining and sublining layers and they are involved in the pathogenesis of RA (49). They can produce matrix metalloproteinases (e.g. MMP-3), which can damage the extracellular matrix and they can also release inflammatory cytokines (like IL-6, IL-1β, TNF-α) (17, 50, 51). Fibroblasts can interact with B cells in the synovium, and fibroblasts can trigger the differentiation and antibody isotype switch by the production of B cell activating factor (BAFF) or a proliferation-inducing ligand (APRIL). Fibroblasts are able to produce GM-

CSF, which can trigger the infiltration of macrophages to the inflammatory sites (17). In addition, fibroblasts can stimulate osteoclastogenesis by the production of receptor-activator of nuclear factor κB ligand (RANKL) that promote osteoclast differentiation and bone erosions (17, 51). When RANKL interacts with RANK, downstream signalling pathways (e.g. NF-κB) will be activated and the process results in osteoclast differentiation and activation (21, 52).

In RA, the synovial fluid contains a high number of neutrophils and these infiltrated cells can mediate tissue damage. Under inflammatory conditions, increased immune complex deposition can be observed in the synovial area (53). In this milieu, neutrophils can bind to these immune complexes by their Fc receptors. Following this interaction, the neutrophils become activated and initiate various effector cell responses, such as the frustrated phagocytosis. This process may trigger the exocytosis of neutrophil granules, leading to the degradation of the extracellular matrix. Additionally, reactive oxygen species (ROS) released from the cells can trigger the oxidative processes (53). The production of ROS by neutrophils can activate some pathogenic signalling-pathways (like the MAPK or NF-κB), while the development of neutrophil extracellular traps (NETs) contributes to the joint destruction in RA (18). Neutrophil matrix metalloproteinase production (like MMP8 or MMP9) have an important role the cartilage degradation. In the inflammatory microenvironment, the neutrophil cytokine- and chemokine production increase the migratory capacity of the cells and enable them to infiltrate the joint cavity more easily (18, 20). Due to the highly diverse effector functions attributed to neutrophils in RA pathogenesis, their development, function, and involvement in the autoimmune process are discussed in a dedicated chapter.

1.3. Neutrophils as important effector immune cells

1.3.1. About neutrophils in general

Neutrophils are generated by hematopoietic stem cells in the bone marrow that differentiate through myeloid progenitors, granulo-monocytic progenitors and unipotent neutrophil progenitors (Figure 3) (54, 55). The main regulator of neutrophil development and release from the bone marrow is the granulocyte colony-stimulating factor (G-CSF) (55-57). Mature neutrophils can be retained in the bone marrow by CXCR4 signalling, stimulated by CXCL12 on stromal cells, meanwhile release from the bone marrow is

stimulated by CXCR2 (55, 58). G-CSF can mediate the process by upregulating the CXCR2 on neutrophils, while downregulating CXCR4 (55). Human CXCR2 ligands (like CXCL1, CXCL2, CXCL5 or CXCL8, which are partially expressed by endothelial cells), can trigger the neutrophil release to the circulation (59, 60). Furthermore, some cytokines can also contribute to neutrophil release, like IL-17, which is partially produced by T cells. IL-17 is able to stimulate granulopoiesis by the activation of G-CSF. It can attract IL-17-producing T cells to the affected area and Th17 cells can recruit more neutrophils, as well (55). Neutrophil recruitment can be increased during inflammation due to high levels of IL-1, through the IL-17 and G-CSF axis by a positive feedback loop (59). Neutrophils are short-lived cells that spend only a few hours in the circulation. During their short lifespan, the phenotype of neutrophils is changing (55).

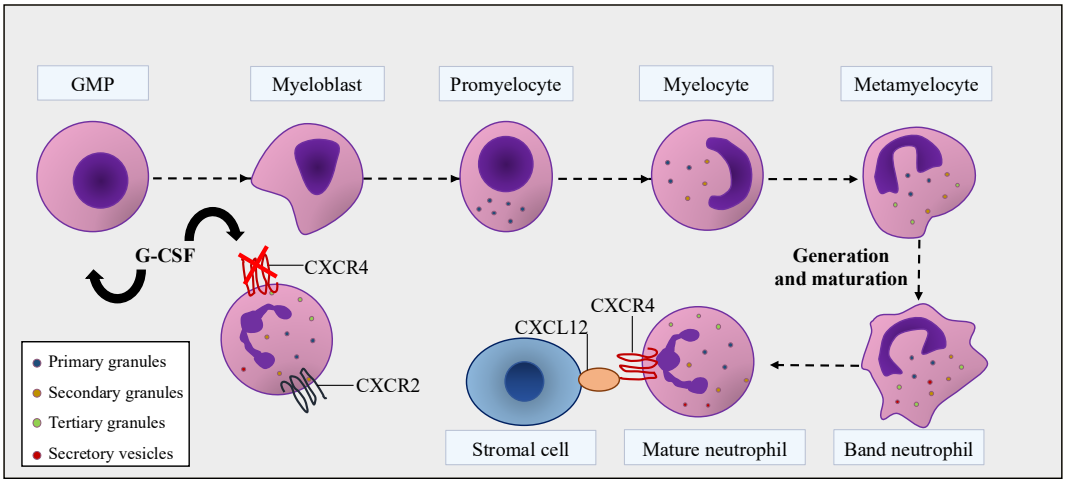

Figure 3: Neutrophil development

Neutrophils originate from granulo-monocytic progenitor cells. The cells develop through myeloblast, promyelocyte, myelocyte, metamyelocyte and band neutrophil phases. The nucleus and cell-specific intracellular granules can be characterized during the development. The main regulator of neutrophil development is G-CSF, which increase the expression of CXCR2 and reduce the expression of CXCR4. CXCL, chemokine ligand; CXCR, chemokine receptor; GMP, granulo-monocytic progenitor (own figure based on reference (55)).

In a mature neutrophil, there are three different granules (the primary, the secondary and the tertiary granules), meanwhile the secretory vesicles of neutrophils have endocytic origin. In these compartments, there are antimicrobial agents (like elastase or myeloperoxidase) and tissue-destructive mediators (like matrix metalloproteinases) (55, 59). Neutrophil effector functions are important in the innate immune system. In the

circulation, neutrophils patrol for signs of microbial infections and if they find them, these cells can quickly respond by killing the invading pathogens (59, 61). They can eliminate the pathogens by phagocytosis, degranulation or the release of nuclear material (by neutrophil extracellular traps (NETs)) (46, 59). Neutrophils play a crucial role in inflammatory processes. Their various special functions may contribute to the pathogenesis of certain human diseases like infections, sepsis, cancer or chronic autoimmune diseases (55).

If they do not participate in inflammatory processes, they can migrate to the liver, spleen or the bone marrow, where they undergo apoptosis and they can be eliminated by resident macrophages (62). Neutrophil ageing is mainly regulated by microbiome fluctuations. Aged neutrophils upregulate, which promote their clearance from the circulation (55). This process is essential in neutrophil homeostasis (62).

1.3.2. Participation in inflammatory processes

Neutrophils can participate in injuries and inflammatory processes, as well (61). Neutrophils can leave the circulating blood and they can reach the affected region or tissue due to stimuli like pro-inflammatory mediators (56). Neutrophils have many cell surface molecules, which can sense the inflammatory signals, such as Fc receptors, adhesion receptors like integrins or selectins, G-protein coupled chemokine receptors or cytokine receptors (55, 63). Numerous pathogen recognition receptors like Toll-like receptors, Nod-like receptors and C-type lectin receptors are also expressed (55). These receptors can activate various intracellular events and cellular responses to trigger the migration (and the activation) of neutrophils (Figure 4). Neutrophil migration is based on complex adhesion and migratory cascades. First of all, neutrophils make an interaction with the endothelial layer by selectins (this stage is called fast rolling). Later they slow down (slow rolling), which is mediated by the interaction between selectins or integrins (LFA-1) and their ligands (61, 64, 65). Next, neutrophils are able to adhere on the endothelial cells by the help of β2-integrins (firm arrest), which is followed by an intraluminal crawling by the help of Mac-1 (55, 64). In addition, neutrophils have to pass through the endothelial barrier. Chemotaxis is regulated by chemoattractants through complement receptors, leukotriene receptors and chemokine receptors (55).

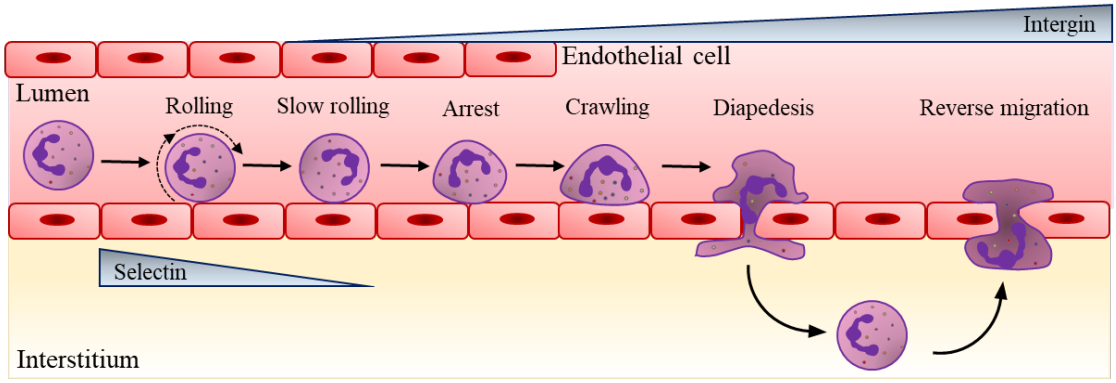

Figure 4: Neutrophil extravasation

Due to infectious or inflammatory stimuli, neutrophils can migrate into the tissues through the vessel wall (extravasation). Neutrophils can make reversible interactions with the endothelial cells by selectins and integrins. They slow down by sensing the inflammatory environment (slow rolling) and they spread over the endothelial cells. After crawling, they will search for the ideal region for trans-endothelial migration by Mac-1 (diapedesis). When they get to the interstitium, they can exert their different effector functions. Neutrophils are able to translocate back to the intravascular space (reverse migration), that may have a role in remote organ damage (own figure based on reference (55)).

Neutrophils can use transcellular and paracellular pathways to migrate through endothelial cells. If they passed the vascular barrier, they have to reach the interstitial space (55). After their successful extravasation, they are able to activate some of their effector functions, which are discussed in the next chapter.

1.3.3. The effector mechanisms of neutrophils

Neutrophils have a critical role in immunity and inflammation and they are able to respond to microbial or pro-inflammatory stimuli. If neutrophils migrate to tissues, they can perform specific effector functions (66). After pathogen recognition, neutrophils eliminate the invading microorganisms by phagocytosis and respiratory burst (55, 67). Respiratory burst is an enzymatic process, which is generated by the NADPH oxidase Nox2 (55). Neutrophil hydrolytic enzymes (like lysozyme, elastase, antimicrobial peptides or lactoferrin) belong to the neutrophil weaponry (Figure 5) (46, 55, 68). Granule-derived molecules are able to kill the pathogen directly (like by reactive oxygen species or matrix metalloproteinases) or they can generate a positive-feedback loop on neutrophils (e.g. with proteases) (55). Neutrophil extracellular trap formation (NETosis) is a unique neutrophil effector function (55, 66). During NETosis, neutrophils are able to

release their DNA into the extracellular space that form NETs (55, 66, 69). NET contains neutrophil-derived antimicrobial agents and neutrophil DNA. These traps are essential to kill pathogens, but they can participate in autoimmune processes. Activated neutrophils can release various chemokines, cytokines (like CXCL8, CCL3) and lipid mediators (like LTB_4), therefore they can easily take part in immune responses and interact with other immune cells (55).

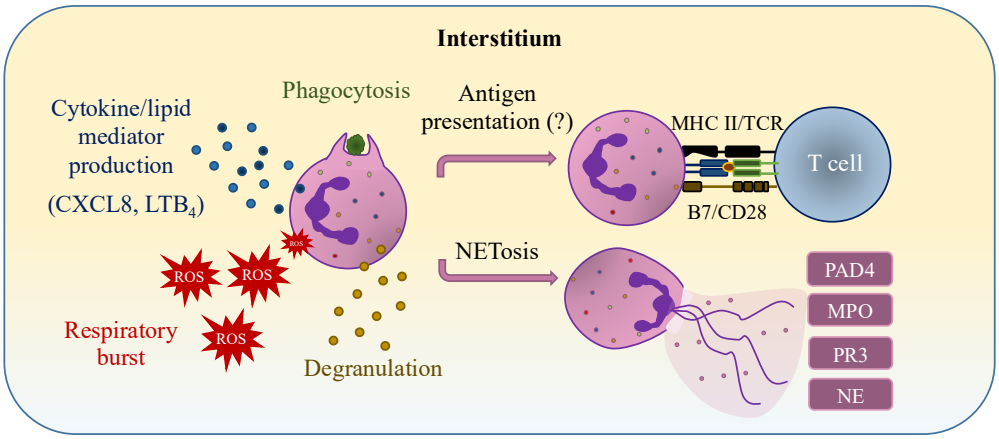

Figure 5: Effector functions of neutrophils

After neutrophils migrated to the inflamed tissue, they utilize their different effector functions (like cytokine or lipid mediator production, respiratory burst, NETosis and phagocytosis or they can take part in antigen presentation to T cells). Effector functions can lead to the elimination of pathogens or they can amplify the inflammatory responses. CXCL, chemokine-ligand; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; PAD4, peptidyl arginine deiminase-4; PR3, proteinase 3; ROS, reactive oxygen species; (own figure based on reference (55)).

Phagocytosis is another antimicrobial mechanism, in which neutrophils recognize the pathogens, which are then enclosed within the phagosome (66, 70). These phagosomes can fuse with cytoplasmic vesicles (containing antimicrobial substances), resulting the formation of phagolysosomes. These structures degrade the invading microbes effectively due to their low pH and the presence of hydrolytic enzymes (59). Under homeostatic conditions, neutrophils do not express molecules on their surface, which are important in antigen presentation, while various pro-inflammatory mediators (e.g. IFN-γ) can enhance this expression (71, 72). This is supported by a previous study, in which treatment of healthy human neutrophils with synovial fluid from rheumatoid arthritis patients enhanced the cell surface expression of HLA class II molecules (71). Antigens that are degraded by neutrophil proteases can associate with HLA molecules in the form of

peptides and with the help of costimulatory factors (like CD80 and CD86 in the presence of increased concentration of IFN-γ), they can participate in antigen presentation (71, 72). In vivo experiments have shown, that the expression of these molecules is enhanced in the presence of memory T cells. This may be due to the fact that memory T cells are capable of producing a significant proportion of IFN-γ (71). It is important to emphasize that although neutrophils are capable to activating T cells, this activation is not as strong as when the presentation is carried out by professional antigen-presenting cells (71). Nevertheless, it is important to highlight that antigen presentation by neutrophils is a relatively new and uncertain theory.

In order to better understand the routes leading to the effector mechanisms of neutrophils, it is necessary to get familiar with the cell surface receptors of neutrophils.

1.3.4. Neutrophil cell surface receptors

Neutrophil functions are mediated by various activating and inhibitory receptors. Activating receptors are able to stimulate neutrophils or increase their affinity to stimulations. Neutrophil G protein-coupled receptors (GPCRs) are activating receptors that play a prominent role in the recognition of bacterial and mitochondria-derived danger signals (like N-formylated peptides) (55, 63, 73). Chemokine receptors on neutrophils like CXCR1 or CXCR2 interact with their ligands, e.g. with CXCL1, CXCL2 or CXCL8, while C5a receptor sense the complement activation by neutrophils (55). GPCR-dependent signalling takes part in cell polarization, and the activation of cell migration. These GPCRs activate phospholipase Cβ-mediated Ca^{2+} signals and activate phosphoinositide 3-kinase isoforms (55). This intracellular signalling affects the NADPH oxidase activation, the release of anti-microbial components from vesicles, and the prolongation of neutrophil survival.

β2-integrins (like LFA-1 or Mac-1) are indispensable in cell-cell interactions, in cell migration, in the induction of cell spreading or in the interaction between the cell and the extracellular matrix (46). Integrin signalling is divided into inside-out or outside-in signalling (64). Two signals are need during the adherent activation of neutrophils, one inflammatory stimulus for example by TNF-α and another by immobilized proteins (74). Pro-inflammatory mediators provide signals to trigger the transition of integrin activation status from inactive to active form and they can increase the ability and avidity of the

receptor toward their ligand (namely inside-out signalling) (74). Subsequently, a second signal is generated, which can lead to actin cytoskeleton rearrangement and effector cell responses (namely outside-in signalling) (74). In this context, TNF-α serves as an appropriate pro-inflammatory cytokine, while fibrinogen functioning as a β2-integrin ligand containing an Arg-Gly-Asp-(RGD) motif, can effectively model the integrin-ligand-dependent cell responses of neutrophils under in vitro conditions. Neutrophil activation by extracellular matrix proteins through integrins trigger complex intracellular tyrosine kinase signalling pathways with the participation of the Src-family kinases and Syk (55).

Fcγ receptors are important cell surface immunoglobulin-family receptors on neutrophils. They are able to mediate effector mechanisms such as immune complex-mediated cytokine or ROS production or the pathogen elimination by phagocytosis.

On the surface of neutrophils, type I and II cytokine receptors (G-CSFR, GM-CSFR), Toll-like receptors or C-type lectin receptors (e.g. Dectin-1) are also present (55, 63). Besides the activating receptors, neutrophils express inhibitory receptors. Most of them signal through Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs), which can inhibit the activation and fine tune the immune responses. FcγRIIb, SIRPα and Siglec9 are all inhibitory receptors on neutrophils (55). As Fc receptors and integrins play a crucial role in neutrophils under inflammatory conditions and as these receptors were in our focus during our experiments, we provide a detailed overview of these cell surface molecules.

1.4. Fc receptors in general

During adaptive immune responses, antibodies have an essential role in protective and in pathogenic processes. Antibodies contain Fc and Fab regions. With the Fab region, they are able to bind to the antigens and form immune complexes, while with their Fc region, they can connect to Fc receptors and can trigger effector cell responses (75).
Fc receptors are immunoreceptors, which are expressed by myeloid cells and B cells. Fc receptors can mediate positive and negative signals. The FcRα subunit can bind to the antibody, so this region is responsible for the specificity. If the Fc receptor binds to IgA, it is called FcαR; if it binds to IgG, it is called FcγR; if it binds to IgE, it is called FcεR. If the receptor associate with IgM, it is called FcμR (75, 76). In the cytoplasmic region of

the receptor Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) can be present, which can phosphorylate tyrosines and activate kinases or Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) can be found, which can inhibit the activation signals. In some cases, the ITAM region is found on the Fc receptor γ-chain or associated the DAP12 adaptor molecule. Phosphorylated ITAM tyrosines can induce effector functions like phagocytosis, degranulation, superoxide or cytokine release. The type of effector function depends on the cell type. Meanwhile, ITIMs can inhibit cell activation (76).

Immune complexes play an important role in autoimmune arthritis, as they can trigger inflammatory signalling pathways by acting on Fcγ receptors. A more detailed understanding of these receptors is essential for elucidating the pathogenesis of autoimmune arthritis.

1.4.1. Fcγ receptors

Fcγ receptors (FcγRs) recognize IgG antibodies by the Fc region (77). FcγRs are structurally related and they belong to the Ig protein family. In humans there are three main FcγRs subtypes: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), which have different structural and functional properties. Some activating Fcγ receptors (such as human FcγRI, FcγRIIIa) can associate with the ITAM-bearing transmembrane adaptor molecule Fc receptor γ-chain.

Human FcγRI is a high-affinity receptor to monomer IgGs. It has three extracellular immunoglobulin-like domains, which is expressed mainly by activated neutrophils (78). FcγRI associates with the Fc receptor γ-chain, which contains the ITAM region in the cytoplasmic part. FcγRII and FcγRIII can associate with IgG by low affinity (79). FcγRII has activating subtypes, like the FcγRIIa and the FcγRIIc, which contain ITAM motifs in the cytosol (79). FcγRIIa is expressed by myeloid cells (monocytes, macrophages, platelets etc.) and the receptor is important in the phagocytosis of IgG-opsonized molecules, in superoxide release, cytokine production and in antibody-dependent cellular toxicity (ADCC) (80). FcγRIIb is an inhibitory receptor, which have ITIM motifs and it can be found on B cells, monocytes, macrophages, dendritic cells, activated neutrophils, mast cells or basophils (79). FcγRIIb is able to crosslink with the B cell receptor, and can inhibit activating signals (80).While FcγRIIc expression is relatively low on the surface of natural killer cells, the activating receptor can trigger the

antibody-dependent cellular toxicity reaction (81). Another type of Fcγ receptors is the low affinity activating FcγRIII (79). FcγRIIIb is subtype, which is expressed by human neutrophils. It is a glycosylphosphatidylinositol anchored protein without transmembrane and cytoplasmic domains (79).

FcγRs (like FcγRIIb, FcγRIIIa, FcγRIV) can be expressed not only on human cells but also on the surface of mouse cells. The FcγRIIIa is a transmembrane protein that associate with the Fc receptor γ-chain and it is expressed by macrophages, natural killer cells, monocytes and T cells (79). It has a role in antiviral immunity and in antitumor responses (Figure 6) (82).

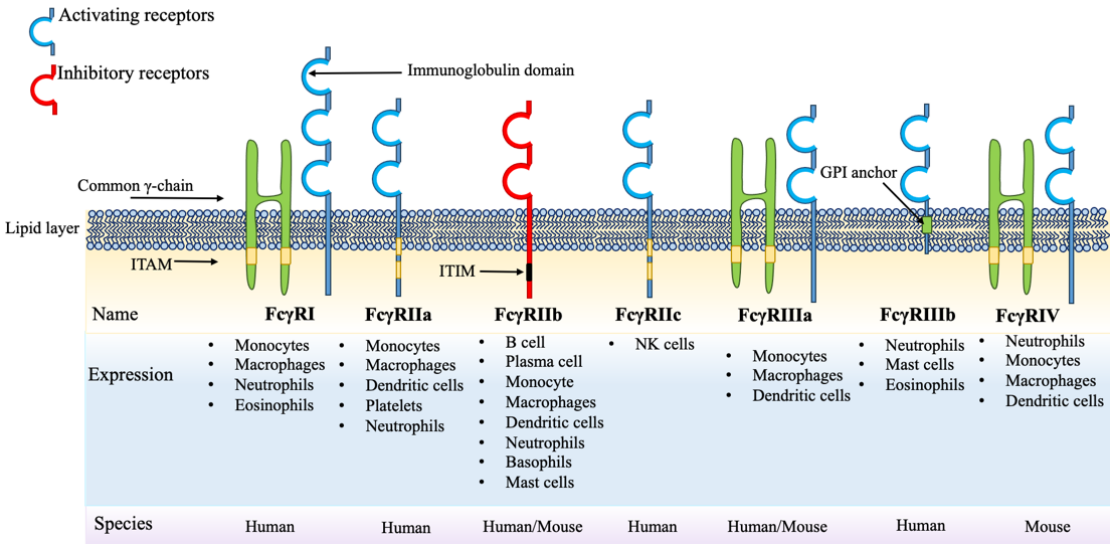

Figure 6: Structure and expression pattern of IgG-specific Fcγ receptors

Fcγ receptors differ in their structure and affinity for IgG antibodies. Activating receptors contain FcR γ-chain associated ITAM regions (like human FcγRI, FcγRIII or mouse, FcγRIV) or the receptor can carry ITAMs independently without the FcR γ-chain (e.g. FcγRIIa, FcγRIIc) in the cytoplasmic domain. Inhibitory receptors (like FcγRIIb) are associated with ITIM sequences. The expression of FcγR subtypes varies between cell types and also differs between species (own figure based on reference (80)).

FcγRIV is a mouse-specific Fc receptor, which is expressed by myeloid cells (like neutrophils, monocytes, macrophages) and it has an important role in ADCC reaction. While the FcγRIV does not have an exact human ortholog, the human FcγRIIIa is functionally similar (83).

1.4.2. Signalling via Fcγ receptors

In the case of receptor activation, the Src family kinases can phosphorylate the FcγR ITAM domain and the associated Syk tyrosine-kinase. Syk is able to activate the downstream signalling by phosphorylating Phosphoinositide 3-kinase or PLCγ isoforms. As a result, the activated inositol trisphosphate stimulates the intracellular release of Ca^{2+} and the Diacylglycerol stimulates Protein kinase C. Activated Protein kinase C has an essential role in membrane rearrangements and actin filament reorganizations through small G-protein stimulation (84). The activating FcγR induced gene expression can also contribute the pro-inflammatory mediator production (TNF-α) (78). The inhibitory FcγRIIb can also be phosphorylated by Src family kinases. This leads to the activation of SHIP phosphatases, which can mediate the dephosphorylation steps, which inhibit the activating signalling pathways. As a results of this inhibition, FcγRIIb can moderate phagocytosis or the expression of pro-inflammatory mediator production. FcγRIIb is the only FcγR, which is expressed by B cells, and if it is cross-linked with the BCR, the antibody production decrease (80). Moreover, it is known that increased autoantibody production can significantly contribute to the formation of immune complexes, which may lead to the development and maintenance of autoimmune inflammatory processes.

1.5. Integrins

Integrins are heterodimer transmembrane glycoproteins, which have multiple conformations and can interact with several ligands. Integrins can be classified according to their α and β chains. They take part in cell-cell interactions (e.g. β1-integrins), in blood clotting mechanisms (e.g. β3-integrins), in cell and extracellular matrix connection (e.g. β4-integrins) or they have a role in the interaction between the mucous membrane and immune cells (e.g. β7-integrins) (85, 86). They can bind to the extracellular matrix through the Arg-Gly-Asp-(RGD) motif of fibronectin, collagen or laminin (87).

β2 integrins have a crucial role in adhesion processes under homeostatic conditions, while increased expression can be detected in the inflamed RA tissue. It has been shown, that the expression of the β2-integrin LFA-1 is indispensable for the development of experimental joint inflammation (88).

Our experiments are prominently conducted in a mouse model of arthritis where integrin-dependent signalling has an important role. Therefore, we provide a detailed overview of β2-integrins.

1.5.1. β2-integrins

β2-integrins also contain α and β subunits. The α chain (CD11) has four subtypes: CD11a, CD11b, CD11c and CD11d. Meanwhile, the β chain (CD18) is a shared chain in β2-integrins. There are four main β2-integrins: LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), αXβ2 (CD11c/CD18) and the αDβ2 (CD11d/CD18). β2-integrins mainly participate in immunological processes, like in neutrophil phagocytosis, ROS production or T cell activation. Integrins can get involved in the formation of adhesion complexes or in the modulation of actin cytoskeleton dynamics, migration, proliferation or apoptosis (89). β2-integrins are expressed mainly by leukocytes. While LFA-1 shows a high expression on most immune cells, Mac-1 is rather found on myeloid cells such as neutrophils, monocytes, macrophages and dendritic cells (87). The CD11c/CD18 is expressed by dendritic cells in mice, while in humans, CD11c/CD18 can be found on natural killer cells and lymphocyte subtypes (87). Higher expression of CD11d/CD18 can be found on human natural killer cells or B cells in inflammatory tissues. β2-integrins have extracellular domains, which have role in ligand binding, while the short cytoplasmic region has regulatory functions (87). The affinity of the receptor depends on the receptor conformation and clustering. There are three main conformational status of integrins: the inactive (without the ligand), intermediate (where ligand-binding is possible with low-affinity) or active (where the integrin is able to bind ligands with high-affinity) (90). Integrins can be found in an inactive and closed conformational state in most of the time. The activation of integrins can be reached by the ligation of cell surface receptors like cytokine receptors. During inside-out signalling talin and kindlin with many other proteins (like α-actinin, filamin A or choronin 1A) can contribute to the functions of integrins through the interaction with the integrin β-chain, which can switch the integrin to active conformation (inside-out signalling, Figure 7). On the other hand, integrins can also trigger the activation of outside-in signalling pathways. The main ligand of LFA-1 is ICAM-1, meanwhile Mac-1 is able to make interactions with ICAM, fibrinogen or the CD40-ligand and it can also recognize the complement C3b fragments (89).

The improper functioning of integrins may promote the onset of pathological processes, such as the Leukocyte adhesion deficiency (LAD) syndrome (63). LAD syndrome is an autosomal recessive disorder, which have three main subtypes. LAD-I is caused by the loss-of function mutation in the CD18 gene and the impaired β2-integrin function (90). The symptoms can be severe bacterial and fungal infections. LAD-I has a sub-variant, the LAD-III disorder, which is characterized by the dysfunction of the Ferritin Family Member 3/kindlin-3, which is important during β2-integrin inside-out signalling (87). In LAD-III, the leukocyte and platelet adhesion functions can be damaged. LAD-II syndrome is often a type IIc glycosylation congenital disorder and the symptoms can be similar as in LAD-I (87). The clinical features of LAD highlight the importance of β2-integrins in immune cell adhesion and migration. To better understand how β2-integrins mediate their functions, the following section focuses on the signalling pathways of β2-integrins.

1.5.2. Signalling of β2-integrins

Small intracellular proteins, like talin and kindlin-3 can mediate the inside-out activation. During the inside-out signalling, the stimulus can trigger conformational changes in the receptor (Figure 7) (90). Talin and kindlin are essential in the rolling of neutrophils on the endothelium (87).

Outside-in signalling can happen after ligand (e.g. ICAM-1) binding (64). Conformational changes (with talin or kindlin-3) will modify the activation status of the integrin. During activation, integrin clustering is promoted by Ca^{2+} and it increases the avidity of the receptor for the ligand (64). Src-family kinases are able to phosphorylate the adapters (like DAP12 or FcRγ chain) on the ITAM motifs that triggers the translocation of Syk. Syk activation can lead to different signalling pathways (like PI3K-Akt or the MAPK cascade). The PI3K-Akt pathway is important for cell survival, migration and inhibition of apoptosis, while the MAPK cascade influences the activation and transcription of inflammatory genes and cell growth (64, 91).

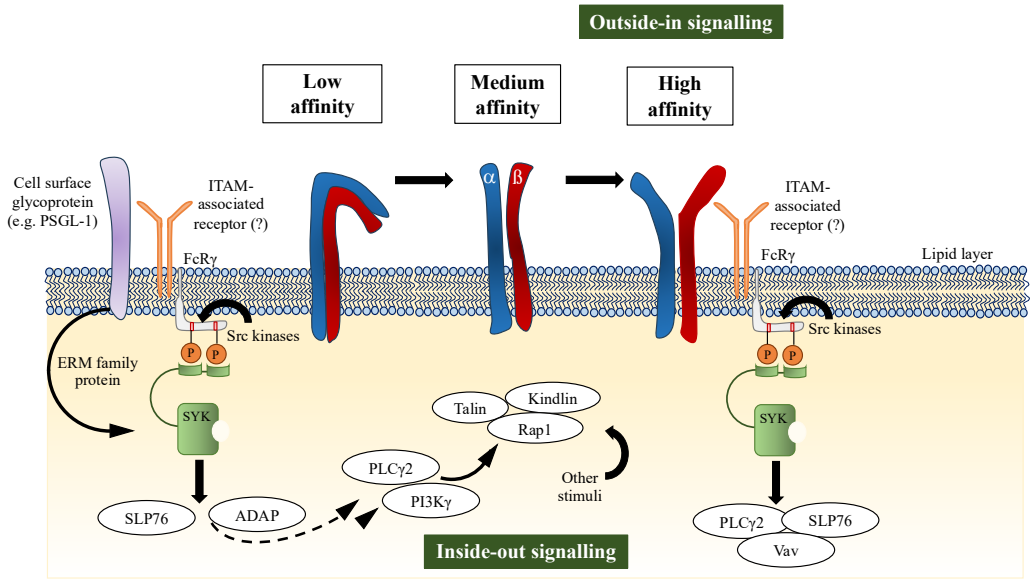

Figure 7: Integrin signalling

Integrins have different conformations, which determine the receptor affinity. Cell surface glycoproteins (like PSGL-1) can activate signalling cascades with the help of adaptors (e.g. SLP76) that can stimulate the switch to medium affinity of the receptor, while talin and kindlin mediate the slow rolling of neutrophils. Integrins can also make connections with ITAM-associated receptors, which are capable of inducing phosphorylation by Src kinases that causes the stimulation of the downstream positioned Syk. ADAP, Adhesion and degranulation promoting adapter protein; PI3Kγ, Phosphoinositide 3-kinase γ; PLCγ2, Phospholipase Cγ2; PSGL-2, P-selectin glycoprotein ligand-1; Rap1, Ras-related protein 1; SLP76, SH2 domain-containing leukocyte protein of 76 kDa; Vav, Vav guanine nucleotide exchange factor (own figure based on reference (63)).

A shared point between Fcγ receptor and β2-integrin is that both of them can use the Syk tyrosine-kinase for their signal transduction.

1.6. Tyrosine kinases

Tyrosine kinases can take part in cell processes like cell differentiation and metabolism, cell growth or apoptosis (92-94). The family of tyrosine kinases can catalyse the phosphorylation of tyrosine residues of target proteins by the help of ATP (95, 96). The target protein can be located in the cytoplasmic region or it can be associated with a receptor intracellular domain. Tyrosine kinases have two main subgroups: receptor tyrosine kinases and non-receptor tyrosine kinases (96). We will focus our attention to non-receptor tyrosine kinases, especially on the Syk tyrosine kinase.

1.6.1. Non-receptor tyrosine kinases

Non-receptor tyrosine kinases are mainly localized in the cytoplasm. Kinase domains are mainly conserved and the catalytic domains are similar to the catalytic domain of the Ser/Thr protein kinases (97, 98). The tyrosine kinase domain is composed of an N-terminal lobe including β sheets and an α-helix and a cytoplasmic C-terminal lobe (which contains α-helixes) (96). In these domains, we can find the ATP biding sites. The C-terminal end activation loop also contains Tyr, Ser or Thr residues. Besides the catalytic domains, the kinases contain non-catalytic domains, as well. The two most well-known non-catalytic domains are the SH2 and the SH3 domains. Non-receptor tyrosine kinases have several groups like the Src-family, the Tec-family, the Abl-family, the Syk, the Csk or the JAK-family members (92, 98). In our experimental arthritis model, we investigated the effect of Syk-selective inhibition, so we give a more comprehensive overview of Syk in the next chapter.

1.6.2. The Syk tyrosine-kinase

Syk is a 72 kDa non-receptor kinase, which was isolated from porcine spleen cells and which has a high expression level in hematopoietic cells (99, 100). It has two SH2 domains at the N-terminal region and one tyrosine kinase domain at the C-terminal end (100). Under homeostatic conditions, Syk has a so-called autoinhibited status. If the interdomains of Syk are phosphorylated or the SH2 domains bound to ITAM regions, it can trigger conformation changes, which can lead to kinase activation (Figure 8). C-type lectin receptors and integrins play a key role in the activation of Syk, which can lead to the activation of the CARD9-Bcl10-MALT1 (caspase-recruitment domain 9–B cell lymphoma 10–Mucosa-associated lymphoid tissue lymphoma translocation protein 1 complex) pathway and the stimulation of the NLRP3 inflammasome. During the receptor activation, Syk is able to trigger the recruitment of molecular-partners, which can increase the downstream signalling pathways and the ITAM phosphorylation by Syk, which can provide a positive feedback loop to the whole signalling procedure (84). Syk has an essential role in the signalling through Fc receptors (like in Fcγ-receptor signalling of neutrophils and macrophages) and β2-integrins.

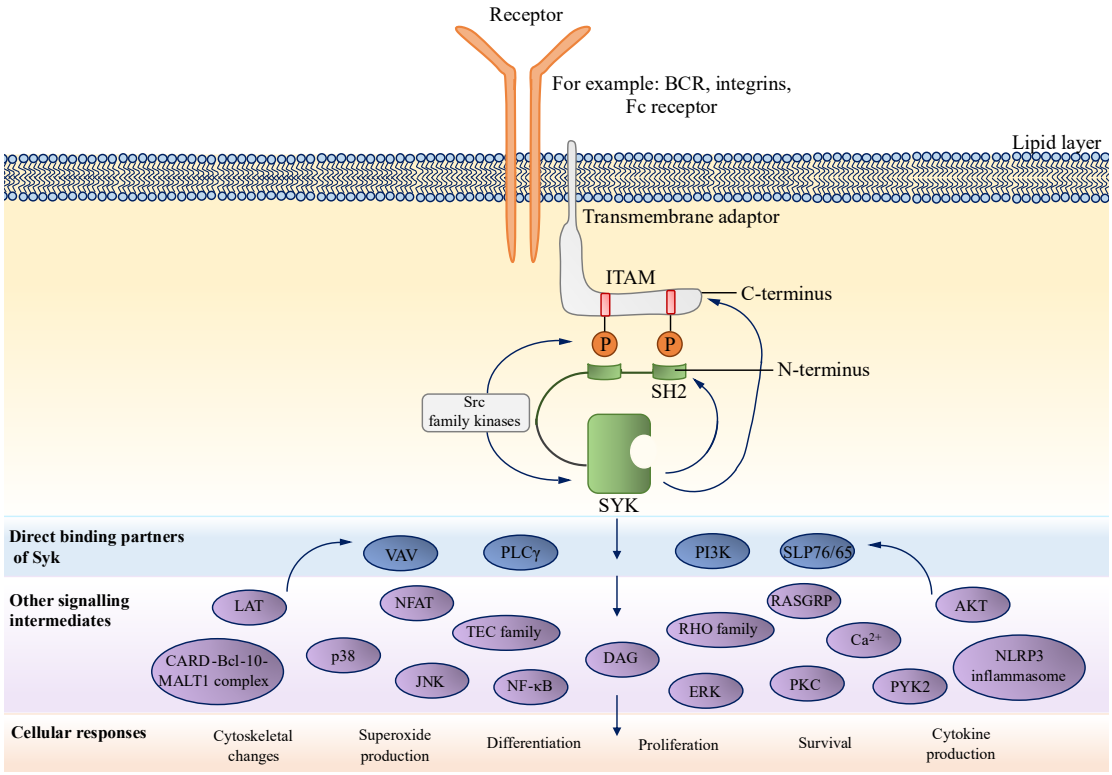

Figure 8: Schematic figure of Syk-associated signal transduction

The tyrosine residues of ITAM can be activated by the Src-family kinases, which promote the recruitment of Syk to the ITAMs that further enhances the activation loop. Syk interacts with several molecules, like Vav, phospholipase-Cγ (PLCγ), the p85α subunit of phosphoinositide 3-kinase (PI3K) and the SH2 domain-containing leukocyte protein 76 (SLP76) or SLP65. These interactions are able to stimulate downstream molecules, while other signalling intermediates (like LAT, p38, NFAT, TEC family members, etc.) can regulate activation of further signalling components. In the end, receptor signalling can trigger cellular responses (own figure based on reference (84)).

Under normal conditions, Syk has an important role in cellular adhesion, osteoclast maturation, platelet activation, B-cell maturation, antifungal processes and in the development of lymph vessels (84, 99).

Meanwhile, Syk can contribute to malignant transformation, or it can participate in some autoimmune processes. Next, we will focus our attention to the role of neutrophils, Fcγ receptors, β2-integrins and Syk in autoimmune arthritis.

1.7. The role of neutrophils, Fcγ receptors, β2-integrins and Syk in autoimmune arthritis

1.7.1. Neutrophils in autoimmune arthritis

Neutrophils have an essential role in anti-microbial immunity, tissue regeneration and repair mechanisms and it has been shown that these cells play a critical role in the pathology of several autoimmune diseases (like rheumatoid arthritis) (101, 102). Their diverse effector mechanisms empower and maintain the inflammation. Neutrophil-derived cytokines, chemokines, respiratory burst, neutrophil extracellular trap formation and antimicrobial peptides can contribute to the autoimmune process (102). They can take part in the initiation and also in the effector phase of autoimmune disease (102). In the synovial area, increased neutrophil accumulation can be detected like in the synovial fluid of the affected joints (55). The increased release of neutrophil ROS in the circulation or in the tissues have been reported in patient with RA (46). The NET formation of neutrophils and NET components (like myeloperoxidase or neutrophil elastase) can directly damage the endothelium and some NET components can also be recognized as autoantigens, which can stimulate the autoantibody production (46). In the inflamed joints, the immune complexes, which are composed by ACPAs or RF, can stimulate neutrophil degranulation through their FcγRs (103). Under inflammatory conditions, neutrophils can produce cytokines and chemokines (e.g. interleukine-8 or TNF), which mediate inflammation and some of them (e.g. IL-1β, TNF-α or IFN-γ) can delay neutrophil apoptosis in vivo (103, 104). Neutrophil-derived interleukin-8 (IL-8) can lead to neutrophil migration from the peripheral blood by a positive feedback loop (105). As the expression of Fcγ receptors and integrins by neutrophils plays a crucial role in arthritis, the following sections will explore the role of these receptors in experimental arthritis.

1.7.2. Fcγ receptors in autoimmune arthritis

In some autoimmune diseases, the deposition of immune complexes (ICs) has a crucial role. The Fc receptor-immune complex interaction can trigger and maintain the pathological inflammatory responses (106). Immune complexes can bind to neutrophils, macrophages or monocytes and can increase some effector cell responses like pro-inflammatory mediator production (62, 106). In autoantibody-induced experimental

models, the activating FcRs (like FcγRI, FcγRIII or FcγRIV) have critical roles, which are associated with the ITAM-bearing transmembrane adaptor molecule Fc receptor γ-chain (102). It has been shown that the lack of FcRγ, led to a total protection from the K/BxN serum transfer arthritis in mice (102, 106, 107). During RA, increased FcγR expression can be measured on circulating monocytes and in the inflamed synovium (108). Moreover, the absence of FcγRIII in mice moderately decreased the severity of joint inflammation (suggesting the contribution of FcγRIV in arthritis), while the lack of FcγRI did not play a critical role in the disease (107). In a previous experiment, it has been shown that the lack of FcγRIV showed a protection from the development of K/BxN serum transfer arthritis (109). In line with this, the local immune cell recruitment was also lower absence of the receptor (109). It has been shown, that when FcRγ was absent only from neutrophils, the mice were completely protected from the development of K/BxN serum transfer arthritis (110). These results suggest an important role for FcRγ-associated Fc receptors on the surface of neutrophils in the development of autoantibody-induced experimental arthritis. As we mentioned earlier, in addition to FcγRs, integrins on the surface of neutrophils also play a prominent role in autoimmune arthritis (110).

1.7.3. β2-integrins in autoimmune arthritis

β2-integrins (like LFA-1 and Mac-1) are also expressed by neutrophils and these receptors can also take part in autoimmune processes (like in autoimmune arthritis) (63, 102). During RA, the increased level of IL-1β in the synovial area can trigger the LFA-1 ligand ICAM1 expression on synovial endothelial cells and the process can increase the influx of immune cells to the synovial tissue (111). β2-integrins have a critical role in experimental autoimmune arthritis. It has been described that the β2-integrin subunit CD18-deficient mice are protected from the development of experimental arthritis (112). Moreover, in mice with CD11a-deficiency, the development of arthritis was not observed, which means that LFA-1 is essential in the onset of autoimmune arthritis (112). It has been shown, that LFA-1 on neutrophils is an important mediator of arthritis development (107). These results with the previously discussed observations indicate an important role for neutrophils and their cell surface receptors (like FcRs and β2-integrins) in autoantibody-induced experimental arthritis.

1.7.4. Syk in autoimmune arthritis

A shared point between Fc receptors and β2-integrins is that both of them use the Syk tyrosine kinase for their signal transduction. Syk can participate in some autoimmune processes like in autoimmune arthritis (100). Syk deficiency results in perinatal lethality due to an impairment in the lymphatic vascular development (113). This problem, can be solved by the creation of bone marrow chimeras with a Syk-deficient ($Syk^{-/-}$) hematopoietic system (113). It has been shown, that in Syk-deficient bone marrow chimeras, the absence of Syk resulted in a total protection from the development of autoantibody-induced arthritis (113). Syk is an important signalling component of neutrophils, platelets or mast cells, so it is an interesting question how cell-specific deletion of Syk affect the development of autoantibody-induced experimental arthritis (102). The neutrophil-specific deletion of Syk nearly totally protected mice from arthritis, suggesting the importance of Syk expression in neutrophils in autoimmune inflammation (99). The mentioned studies indicate that neutrophils, their certain cell surface molecules and the Syk tyrosine kinase play a critical role in the development of autoimmune arthritis. Based on these findings, it would be interesting to test the effect of Syk-selective inhibition on the development of experimental joint inflammation.

1.7.5. Selective Syk inhibition

Over the years, the inhibition of tyrosine kinases became a possibility in the treatment of autoimmune arthritis. Entospletinib is a second generation, Syk-selective blocker (114). Based on the kinase profile of entospletinib, the inhibitor interacts with only one other kinase, namely the tyrosine kinase non receptor 1 at a low concentration (114). Tyrosine kinase non receptor 1 mainly takes part in foetal development, so we can assume that entospletinib exerts its inhibitory effect mainly through inhibiting the activity of the Syk tyrosine kinase (114). Entospletinib has a tolerable safety profile according to hematological clinical trials in patients with chronic lymphoid leukemia and an important advantage of the inhibitor that it can be administered orally (114).

Consequently, it is worth to test the effect of entospletinib in a Syk-dependent experimental autoimmune arthritis.

2. Objectives

During our experiments, we were interested in the following questions:

1) How pharmacological inhibition of Syk influences the development of autoantibody-induced experimental arthritis?

2) How entospletinib affects the leukocyte infiltration to the joints and the in vivo pro-inflammatory mediator production?

3) What is the effect of Syk inhibition on Fcγ receptor- and β2-integrin-mediated effector cell responses of mouse neutrophils?

4) How Syk-selective inhibitors act on the immune complex-triggered cell functions of human neutrophils?

3. Methods

3.1. Experimental animals[1]

KRN T cell-receptor heterozygous transgene animals were mated with wild type C57BL/6 mice, NOD mice were purchased from the Jackson Laboratory (106). Wild type C57BL/6 mice were purchased from the Hungarian National Institute of Oncology or received as a kind gift from the Institute of Translational Medicine, Semmelweis University (Ref. I.). The animals were kept in individually ventilated cages in a conventional animal facility, while the food and water were supplied *ad libitum* (115). All animal experiments were approved by the Animal Experimentation Review Board of Semmelweis University (99).

3.2. K/BxN serum transfer arthritis

KRN T cell receptor transgene mice were mated with NOD mice to it achieve transgene-positive (arthritic) K/BxN and transgene-negative (non-arthritic) BxN mice (116-118). To check the presence of the transgene, we carried out allele-specific PCR (Ref. I.). The arthritic and non-arthritic sera were taken by retroorbital bleeding (Figure 9) (99).

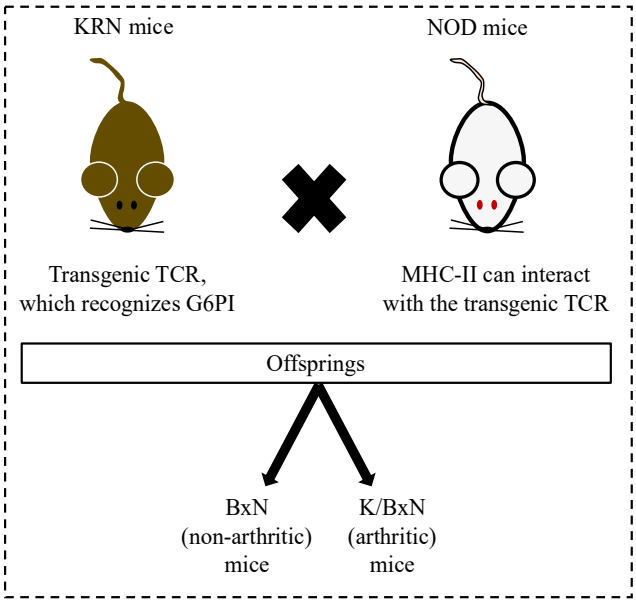

Figure 9: The K/BxN arthritis model
KRN mice carry a transgenic T cell receptor recognizing glucose-6-phosphate isomerase, while non obese diabetic (NOD) mice express autoimmune prone MHC Class II molecules. In the first generation, a subset of the animals, namely K/BxN mice spontaneously develop arthritis. K/BxN arthritic and BxN non-arthritic serum was prepared via retro-orbital blood collection. G6PI, glucose-6-phosphate isomerase; TCR, T cell-specific receptor (own figure based on reference:(119)).

Arthritis was induced by an intraperitoneal injection of 300 µl K/BxN (arthritic) serum. Mice receiving BxN serum served as controls (Ref. I.). The severity of joint inflammation

[1] *The labels Ref. I. or Ref. II. in the "Methods and Results" chapters refer to the Candidates publications (see Chapter 9.).*

was detected by ankle thickness measurement with a spring-loaded caliper, while as relative measurement, the clinical scores was determined by two investigators on every day on a 0-10 scale (117, 120). Clinical scoring was based on subjective observations and on the classical signs of inflammations (e.g. oedema, redness). The maximum score was given for the most severe joint inflammation.

3.3. Oral administration of the vehicle and the inhibitor

Entospletinib (GS-9973, from Selleckchem) was diluted in water and mucilage, while vehicle treatment contained only water and mucilage. Mice were treated twice a day by gavage, and the treatment begun one day before the induction of K/BxN serum transfer arthritis (Ref. I.).

3.4. Flow cytometry analysis

Neutrophil and monocyte numbers were detected in the peripheral blood of vehicle- or entospletinib-treated mice by the labelling with anti-Ly-6G and anti-CD11b antibodies (PE-conjugated, clone 1A8 and biotin-conjugated[2], clone M1/70, from BD Biosciences). The cells were identified on the basis of forward and side scatter characteristics by flow cytometry. Ly-6G- and CD11b-positive cells, were determined as neutrophils, while the Ly-6G-negative and CD11b-positive cells were identified as monocytes (Ref. I.).

We also measured the number of local infiltrated immune cells in the joints. Arthritic and non-arthritic (control) mice were sacrificed and the hind and fore paws were digested by liberase (from Sigma) (121). The local numbers of neutrophils and macrophages were determined by their Ly-6G- and CD11b-expression pattern (121).

The expression of isolated mouse neutrophil cell surface markers was followed by anti-Ly-6G (PE-conjugated, clone 1A8), anti-FcγRII/III (clone 2.4G2)[3], anti-FcγRIV (APC conjugated, clone 9E9), anti-CD11a (biotin-conjugated, clone M17/4)[2], anti-CD11b (biotin-conjugated, clone M1/70)[2] or anti-CD18 (biotin-conjugated, clone C71/16)[2] antibodies (all from BD Biosciences) Cell surface expression levels were measured by flow cytometry (Ref. I.).

---

[2]Biotin-conjugated antibodies were visualized by PerCP-Streptavidin (from BD Biosciences).

[3]It was visualized by a polyclonal Goat-anti mouse-FITC antibody (from Nordic MuBio).

3.5. In vivo measurement of synovial fibroblast numbers and activation

After liberase (from Sigma) digestion of the limbs, we detected the number of local synovial fibroblasts by the help of a specific cell surface marker (by an anti-CD90.2-PE antibody, clone 30-H12, from BD Biosciences). We also measured the activation status of these cells by an anti-MHC Class II-FITC antibody (clone M5/114.15.2, from Millipore), meanwhile the intracellular tyrosine-phosphorylation was detected by an anti-phosphotyrosine antibody (clone PY20, from Southern Biotech) (Ref. I.). For the intracellular staining of fibroblasts, we had to fix and permeabilize the cells with fixation and permeabilization buffers (all from Invitrogen by Thermo Fisher) and we measured the samples by flow cytometry (Ref. I.).

3.6. In vitro analysis of neutrophil cell responses

In our in vitro experiments, mouse neutrophils were isolated from the femurs and tibias (Ref. I. and Ref. II.). We washed out bone marrow from the bones by Hanks' Balanced Salt Solution (without Ca, Mg and Phenol Red, from Capricorn) supplemented with 20 mM HEPES (from Sigma), and the bone marrow red blood cells were lysed by a hypotonic buffer. Neutrophils were separated by Percoll (from GE Healthcare) gradient centrifugation (117, 121, 122). Human neutrophils were isolated (after the volunteers provided written and informed agreement, the Human ethics approval number was 31937-7/2020EÜIG) from the peripheral blood from healthy volunteers (Ref. II.). After the blood collection (in the presence of heparin), plasma was separated and human neutrophils were isolated by Ficoll (from GE Healthcare) centrifugation (Ref. II.). The red blood cells were lysed with a hypotonic NaCl solution (123).

Nunc MaxiSorp F96 plates (from Thermo Fisher) were coated with immobilized immune complexes or integrin ligands, while the functionality of neutrophils was tested by the protein kinase C activator Phorbol 12-myristate 13-acetate (PMA) (from Sigma) (Ref. I.). Immobilized immune complexes were generated by coating the plate with human serum albumin (from Sigma) in a 20 μg/ml concentration for one hour, after that, the wells were blocked with PBS 10% FCS and then were treated with anti-human serum albumin antibody (from Sigma) (in a 1:400 dilution) (124, 125). In some cases, we coated the plate with His-tagged murine Type-VII collagen, at a 2 μg/ml concentration for one hour, after that, the wells were blocked with PBS 10% FCS and then were treated with

rabbit anti-CVII polyclonal (IgG) antibody (at a 1:2000 dilution) (115). To measure the integrin-mediated cell responses, we coated the plate with fibrinogen (from Sigma) in a 150 μg/ml concentration as a ligand of β2-integrins and we used mouse TNF-α (from Peprotech) as a pro-inflammatory stimulus (125). To detect the effect of the inhibitors, we incubated the cells in the presence of entospletinib (GS-9973, from Selleckchem) or vehicle (dimethyl-sulfoxide in 0.01 % concentration), while human neutrophils were preincubated with vehicle (dimethyl-sulfoxide in 0.01 % concentration), entospletinib (GS-9973, from Selleckchem) or lanraplenib (GS-9876, from Selleckchem) for 10 minutes in 37°C (Ref. I. and Ref. II.). After the incubation step, we stimulated the cells and measured the superoxide release by a cytochrome c-reduction assay and during the cell spreading we took photos of the cells by phase contrast microscopy (at 20x times magnification). On immune complex or integrin-ligand surface, neutrophils underwent morphological changes and became darker and the number of the spread cells were measured by ImageJ programme. Cytokine production was measured by the help of commercial ELISA kits (from R&D Systems) after a 6 hour incubation (117, 121). IC_{50} values were calculated by a non-linear regression analysis of dose-response curves or diagrams (Ref. I. and Ref. II.).

3.7. Quantification of pro-inflammatory mediator levels in the joints

To quantify the inflammatory mediator production in the joints, we washed out the joints with PBS supplemented with 10 mM EDTA (pH 7.5) and 20 mM HEPES (from Sigma) (Ref. I.). The levels of pro-inflammatory mediators in the supernatants were measured by commercial ELISA kits (from R&D Systems) (117, 121).

3.8. Statistical analysis

The experiments were repeated for the indicated number of times. For statistical analysis, we used the STATISTICA program (Ref. I. and Ref. II.). Two- or three-way ANOVA was carried out (where inhibitor-, arthritic serum-treatment or in vitro stimulation ± time were the independent variables). The diagrams and curves show mean and SEM from all independent *in vitro* experiments or from individual mice of different *in vivo* experiments. Area under the curve (AUC) was used for kinetic measurements. P-values below 0.05 were considered as statistically significant (Ref. I. and Ref. II.).

4. Results

4.1. The Syk-selective inhibitor entospletinib decreased the macroscopic signs of autoantibody-induced experimental arthritis

In the pathogenesis of RA many cell types are involved like neutrophils, monocytes, macrophages or synovial fibroblasts. First, we wanted to test how entospletinib effects the peripheral and synovial cell numbers under non-arthritic conditions. We saw that entospletinib-treatment did not decrease the bone marrow and the peripheral number of neutrophils (Fig. 10a-b; p=0.88 and p=0.89, respectively) in control serum-treated animals (Ref. I.). In agreement with these observations, the number of monocytes in the peripheral blood and the synovial macrophage and fibroblast numbers did not decrease in the presence of the inhibitor, compared to the vehicle-treated groups (Fig. 10c-e; p=0.99, p=0.84 and p=0.45, respectively) under non-inflammatory conditions (Ref. I.).

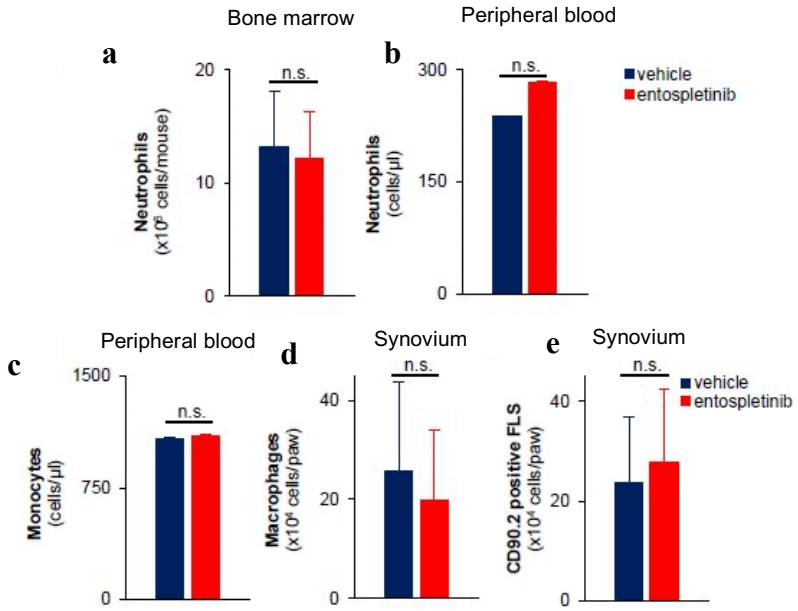

Figure 10: The effect of entospletinib on bone marrow, peripheral and synovial cell numbers (under non-inflammatory conditions)

The Syk-selective inhibitor entospletinib did not decrease the neutrophil numbers in the bone marrow (a) and in the peripheral blood (b). The number of peripheral monocyte (c), synovial macrophage (d) and fibroblast (e) numbers also did not decrease in the entospletinib-treated mice compared to vehicle-treated animals. (a) Cell/mouse dimension refers to bone marrow, which was isolated from two femurs and two tibias. Diagrams show mean and SEM from 3 independent experiments. FLS, synovial fibroblast; n.s. statistically not significant (Ref. I.).

Next, we wanted to test how entospletinib effects the progression of mouse joint inflammation. During our *in vivo* studies, we administered entospletinib or vehicle orally twice a day (Fig. 11a-c and Fig. 12a-c) (Ref. I.).

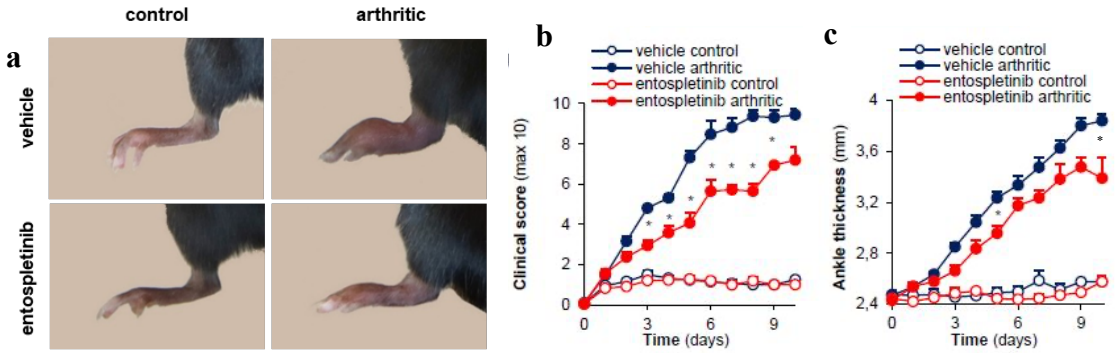

Figure 11: The lower entospletinib dose moderately reduced the severity of joint inflammation

*The oral administration of 50 mg/kg entospletinib moderately decreased the severity of joint inflammation (a-c). Representative images (a) or mean and SEM (b-c) are shown from 11 control and 13-14 entospletinib-treated animals from 5 independent experiments. *p<0.05. (Ref. I.)*

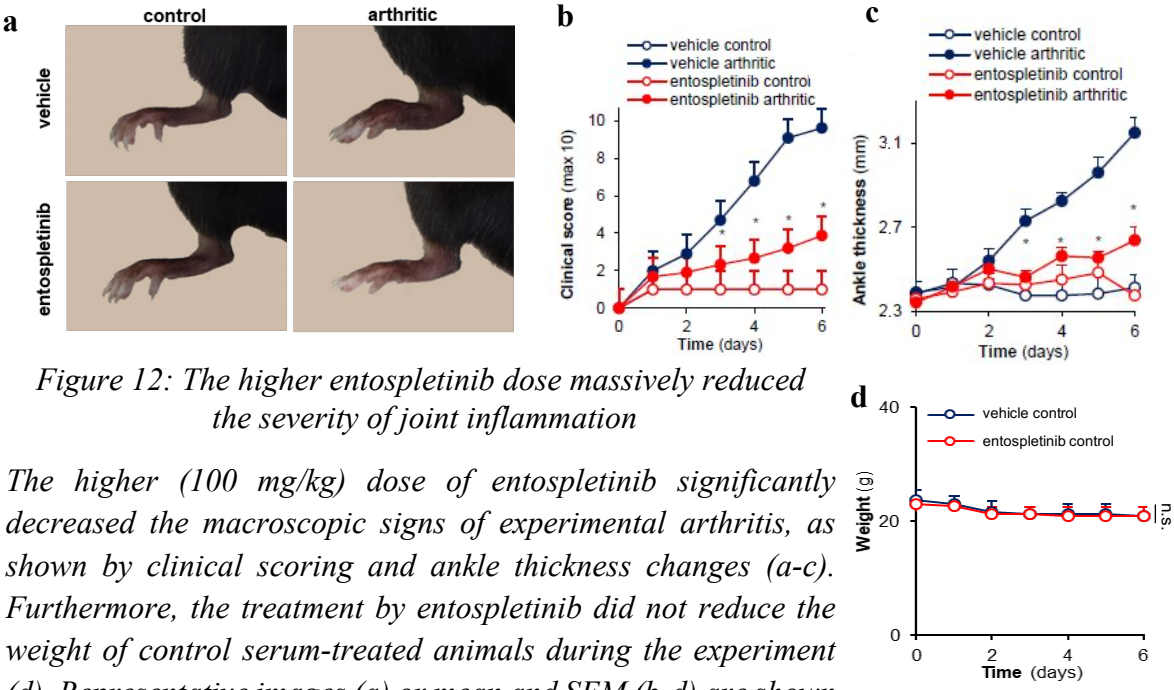

Figure 12: The higher entospletinib dose massively reduced the severity of joint inflammation

*The higher (100 mg/kg) dose of entospletinib significantly decreased the macroscopic signs of experimental arthritis, as shown by clinical scoring and ankle thickness changes (a-c). Furthermore, the treatment by entospletinib did not reduce the weight of control serum-treated animals during the experiment (d). Representative images (a) or mean and SEM (b-d) are shown from 3 control and 5-6 entospletinib-treated animals from 3 independent experiments. *p<0.05. (Ref. I.)*

In a 50 mg/kg dose, entospletinib moderately reduced the severity of joint inflammation, (Fig. 11 a-c).

When we administered a higher entospletinib dose twice a day, the inhibitor massively reduced the severity of joint inflammation (Fig. 12a-c) (Ref. I.). To check the tolerability of entospletinib, we looked for the visible health problems, and also measured the weight of the mice, which did not decrease in the vehicle and in the entospletinib-treated groups in the control serum-treated animals (Fig. 12d, p=0.99).

To sum it up, the Syk-selective inhibitor entospletinib could dose-dependently decrease the severity of autoantibody-induced experimental arthritis (without affecting the number of peripheral or synovial cells under non-inflammatory conditions) (Ref. I.).

4.2. The effect of entospletinib on the local cell numbers

In the following experiments, we tested how entospletinib-treatment affected the numbers of local cells after arthritis induction (Fig. 13a-c). As our results show, entospletinib significantly reduced the number of local neutrophil numbers at both concentrations, compared to the vehicle-treated samples (Fig. 13a, p=0.048 for the 50 mg/kg and p=1.9x10^{-4} for the 100 mg/kg dose, respectively) (Ref. I.). Meanwhile, entospletinib did not reduce the recruitment of macrophages (Fig. 13b, p=0.07 for the 50 mg/kg and p=0.065 for 100 mg/kg dose) or the number of synovial fibroblasts (Fig. 13c, p=0.45 for the 50 mg/kg and p=0.42 for the 100 mg/kg dose) (Ref. I.).

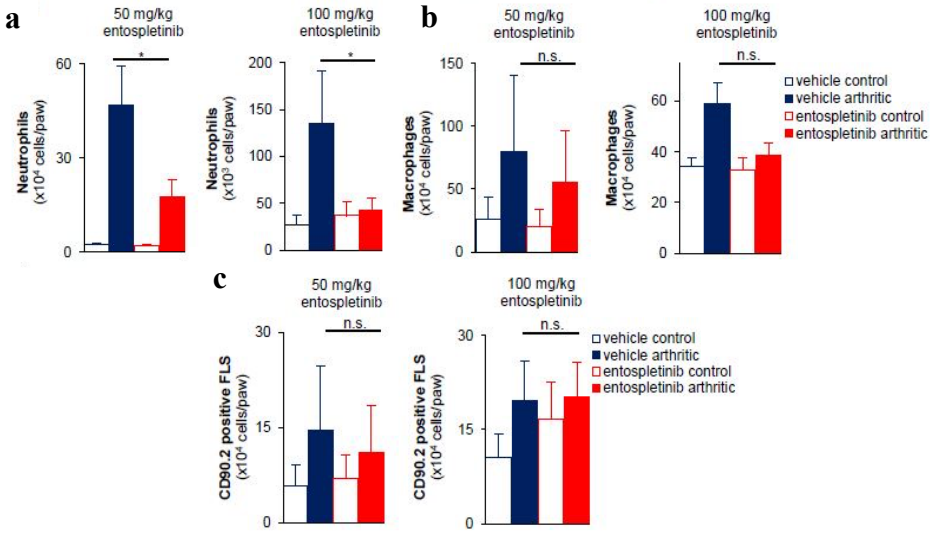

Figure 13: The effect of entospletinib on cell numbers after arthritis induction

*The lower and the higher doses of entospletinib significantly reduced the local neutrophil numbers in the joint (a), while entospletinib did not decrease the presence of synovial macrophages (b) or fibroblasts (c). Diagrams show mean and SEM from 3-5 independent experiments. FLS; synovial fibroblast; n.s. statistically not significant. *p<0.05. (Ref. I.).*

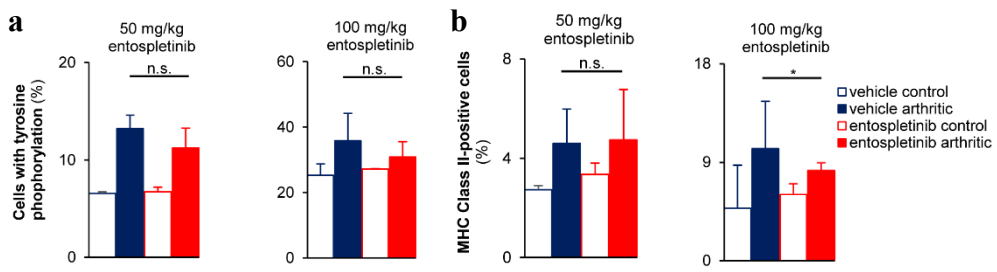

Figure 14: The effect of entospletinib on synovial fibroblast activation

*While the inhibitor did not decrease the intracellular tyrosine phosphorylation of synovial fibroblasts (a), 100 mg/kg entospletinib-treatment significantly decreased the cell surface MHC Class II expression (b). Diagrams show mean and SEM from 3-5 independent experiments; n.s. statistically not significant. *p<0.05. (Ref. I.).*

We also checked the activation status of synovial fibroblasts. After the identification of the synovial fibroblasts by their specific CD90.2 cell surface marker, we labelled them by anti-MHC Class II antibodies and after permeabilization and fixation steps, we measured the intracellular tyrosine phosphorylation of the cells (by an anti-phosphotyrosine antibody). The intracellular tyrosine phosphorylation of fibroblasts did not decrease significantly in the entospletinib-treated samples (Fig. 14a, p=0.57 for the 50 mg/kg and p=0.084 for the 100 mg/kg dose) (Ref. I.). Synovial fibroblasts can participate in antigen presentation by their MHC Class II molecules. Under inflammatory conditions, the expression level of MHC Class II can be increased. We found that the expression of MHC Class II significantly decreased in mice treated with a higher dose of entospletinib (Fig. 14b, p=0.99 for the 50 mg/kg and p=0.034 for the 100 mg/kg dose) (Ref. I.).

Taken together, we saw that entospletinib did not affect significantly the number of local macrophages and synovial fibroblasts, while the inhibitor significantly reduced the recruitment of neutrophils after arthritis induction (Ref. I.). This proposes that neutrophil-dependent mechanisms can stand behind the macroscopic phenotype.

4.3. Pro-inflammatory cytokine levels in the synovial area

In the pathogenesis of RA, several pro-inflammatory cytokines are important. Here, we tested the local levels of MIP-1α (CCL3), MIP-2 (CXCL2, which is an analogue of human IL-8) and IL-1β (Fig. 15a-c) (Ref. I.).

We found that in vehicle-treated arthritic samples, the levels of inflammatory cytokines and chemokines were dramatically increased, while entospletinib-treatment significantly decreased the concentrations of local MIP-1α (Fig.15a, $p=1.6 \times 10^{-4}$), MIP-2 (Fig 15b, $p=1.6 \times 10^{-4}$) and IL-1β (Fig. 15c, $p=1.6 \times 10^{-4}$) at the site of inflammation (Ref. I.).

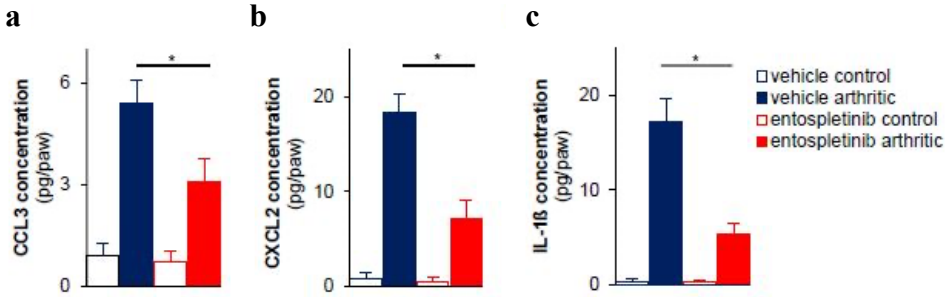

Figure 15: Entospletinib-treatment reduced the inflammatory mediator production under in vivo conditions

*Entospletinib-treatment significantly decreased the local cytokine production compared to vehicle-treated groups (a-c). Diagrams show mean and SEM from 3-4 independent experiments. *p < 0.05. (Ref. I.).*

4.4. Immune complex-mediated cell responses of neutrophils

Next, we tested if entospletinib had any effects on the in vitro neutrophil cell responses. First, we investigated the impact of entospletinib on mouse neutrophil maturation and Fcγ receptor expression (Fig. 16). We found that, entospletinib did not reduce the expression of the maturation marker Ly-6G or FcγRII/III and FcγIV, meaning that the used bone marrow mouse neutrophils were mature and their Fcγ receptor expression were intact (Ref. I.).

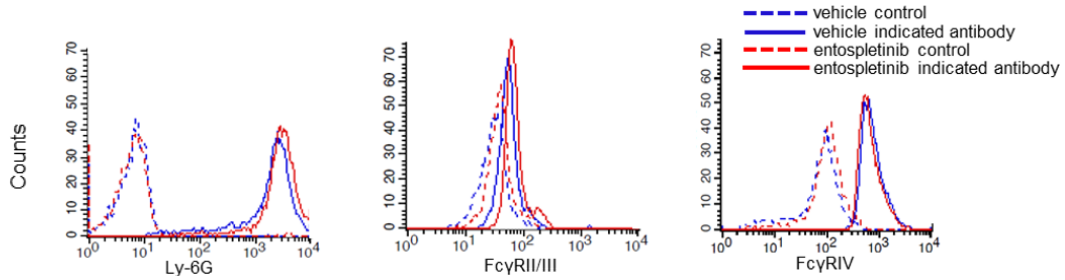

Figure 16: The effect of entospletinib on mouse neutrophil maturation and FcγR cell surface expression

Entospletinib did not affect neutrophil maturation and FcγR expression. Representative histograms from 3 independent experiments are shown. (Ref. I.).

In order to validate our in vivo observations, we carried out in vitro experiments. We generated immobilized immune complex surfaces and we checked some effector cell responses, like superoxide release, cell spreading and cytokine production (Ref. I.). Vehicle-treated neutrophils released high amounts of superoxide on immune complex surface, while entospletinib-treatment dose-dependently decreased the production of toxic oxygen intermediates (Fig. 17a, p=0.017 for veh. vs. 1 µm ento.) (Ref. I.). To validate that neutrophil functionality was not influenced by the inhibitor, we stimulated neutrophils by the protein kinase C activator PMA and we observed that entospletinib did not decrease the superoxide release of the cells at any used concentrations (Fig. 17b, p=0.99 for veh. vs. 10 µm ento.). We also investigated neutrophil cell spreading and we found that entospletinib-treatment dose-dependently decreased the number of activated cells (Fig. 17c-d, p=2.3 x 10^{-4} for veh. vs. 1 µm ento., and p=0.047 veh. vs. 0.1 µm ento.) (Ref. I.).

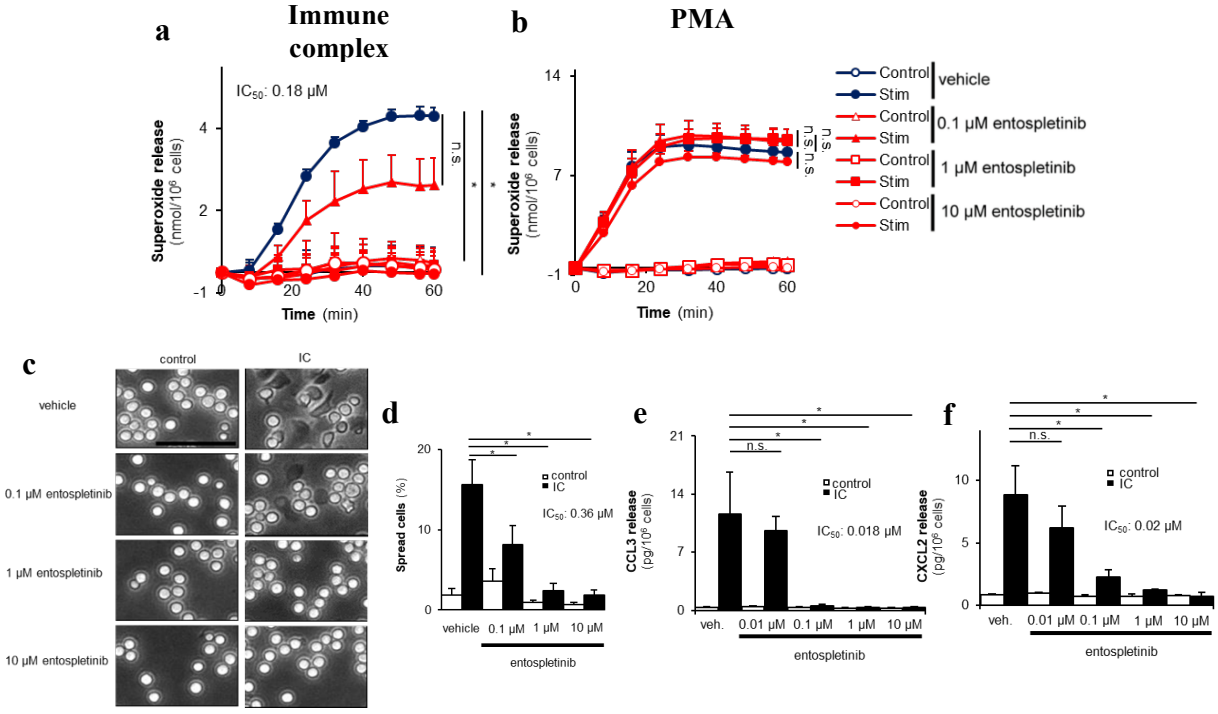

Figure 17: Entospletinib dose-dependently decreased the cell responses of immune complex activated neutrophils

*Entospletinib dose-dependently decreased the immune complex-triggered superoxide production (a), cell spreading (c-d) and cytokine release (e-f). Entospletinib did not decrease the PMA triggered superoxide release of neutrophils (b). Representative pictures (c) from 3 independent experiments are shown. Diagrams (a-b, d-f) show mean and SEM from 3 independent experiments. IC, immune complex; PMA, Phorbol 12-myristate 13-acetate; n.s., statistically not significant; Stim., stimulated; veh., vehicle; *p<0.05; scale bar: 50 µm. (Ref. I.).*

When we checked the inflammatory cytokine and chemokine production, we saw that on immune complex surfaces, vehicle-treated neutrophils released high amounts of inflammatory mediators, while inhibitor-treatment effectively decreased this immune complex-mediated effector cell response (Fig. 17e-f, $p=8.6 \times 10^{-3}$ veh. vs. 0.1 μm ento., $p=1.1 \times 10^{-3}$ veh. vs. 0.1 μm ento.) (Ref. I.).

Next, we wanted to validate the effect of entospletinib on another immune complex surface. In contrast to the vehicle-treated samples, the second generation Syk-selective inhibitor entospletinib dose-dependently reduced the Type-VII Collagen (CVII) - anti Type-VII Collagen (anti-CVII) antibody immune complex-stimulated neutrophil superoxide release (Fig. 18a, $p=1.7 \times 10^{-4}$ veh. vs. 1 μm ento.) and cell spreading (Fig. 18b, p=0.016 veh. vs. 0.1 μm ento.) (Ref. II.).

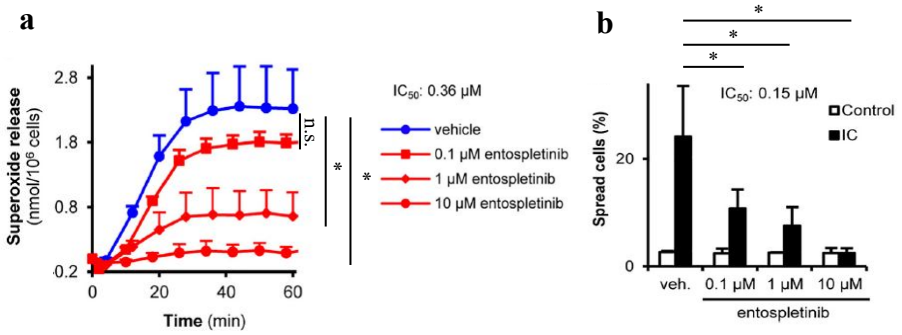

Figure 18: The Syk-selective entospletinib also reduced the effector cell responses on CVII-anti-CVII immune complex surface

*The pharmacological inhibition of Syk dose-dependently reduced the superoxide release (a) and cell spreading of granulocytes (b). Curves (a) and the diagram (b) show mean and SEM from 3 or 6 independent experiments (control datas were subtracted on panel 18a), IC, immune complex; veh., vehicle. *p<0.05. (Ref. II.)*

Overall, we found that entospletinib effectively decreased the Fcγ receptor-mediated cell responses, while maturation and FcγR expression was unaffected.

4.5. Integrin-dependent neutrophil cell responses in the presence of entospletinib

Integrins have a critical role in the development of autoimmune arthritis. First, we checked how entospletinib influenced the cell surface expression of integrin α and β chains on isolated neutrophils. Our histograms show that entospletinib-treatment did not decrease the expression levels of β2-integrin components like CD11a, CD11b or CD18, compared to vehicle-treated samples (Fig. 19) (Ref. I.).

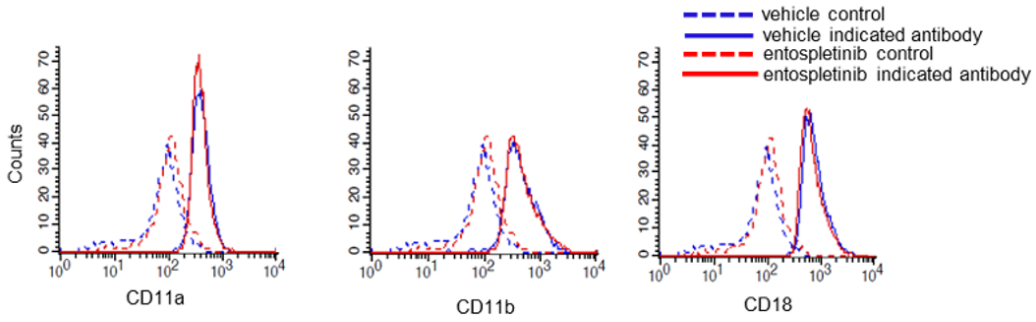

Figure 19: The effect of entospletinib on neutrophil β2-integrin α and β chain expression patterns

Entospletinib-treatment did not decrease the expression levels of integrin α and β chains. Representative histograms from 3 independent experiments are shown (Ref. I.).

Next, we tested how entospletinib affected the effector cell responses on integrin ligand surfaces, so we plated the freshly isolated neutrophils on fibrinogen surface in the presence of TNF-α (Fig. 20 a-f) (Ref. I.).

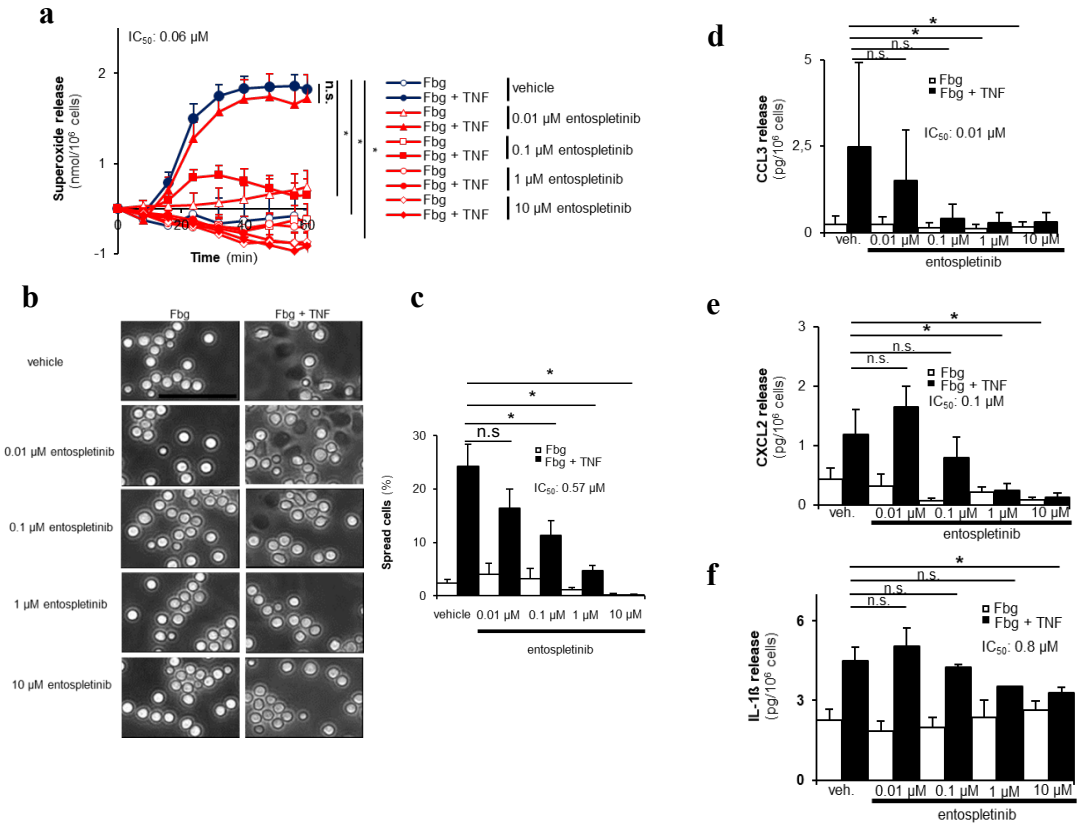

Figure 20: Entospletinib decreased the integrin-mediated neutrophil cell responses

*Entospletinib dose-dependently decreased the fibrinogen activated neutrophil superoxide release (a), cell spreading (b-c) and cytokine production (d-f) in the presence of TNF-α. Phase contrast microscopic images (b) from 3 independent experiments are shown. Panels (a,c-f) show mean and SEM from 3-5 independent experiments. Fbg, fibrinogen; n.s. statistically not significant; veh., vehicle; *p<0.05; scale bar: 50 μm. (Ref. I.).*

Integrin ligand-stimulated, vehicle-treated neutrophils produced high amounts of superoxide and nicely spread over the surface, which was dose-dependently decreased in the presence of the inhibitor (Fig. 20a and Fig. 20b-c, p= 3.5 x 10^{-3} veh. vs. 0.1 μm ento., and p= 0.004 veh. vs. 0.1 μm ento., respectively) (Ref. I.). Next, we checked how entospletinib influenced the inflammatory mediator production of the cells. While in the supernatant of immune complex-stimulated, vehicle-treated neutrophils, the level of CCL3, CXCL2 and IL-1β robustly increased, entospletinib reduced the production in a dose-dependent manner (Fig. 20d-e, p=0.013 and p=6.6 x 10^{-4} veh. vs. 1 μm ento. and Fig. 20f, p=0.029 veh. vs. 10 μm ento., respectively) (Ref. I.).

To summarize this part, we can say that entospletinib decreased the integrin-mediated neutrophil cell responses, which supports the theory that entospletinib may also have the ability to reduce the neutrophil β2-integrin-mediated effector functions under in vivo conditions.

4.6. The effect of Syk-selective inhibitors on human neutrophil cell responses

To strengthen the translational aspects of our experiments, we tested how entospletinib affects human neutrophil activation (Fig. 21a-c). We isolated granulocytes from human peripheral blood and we tested the superoxide production and cell spreading on an immobilized immune complex surface. While vehicle-treated human neutrophils had increased CVII-anti-CVII immune complex activated neutrophil superoxide production and cell spreading, the inhibitor reduced it in a dose-dependent manner (Fig. 21a, p=2.9 x 10^{-3} veh. vs. 1 μm ento., Fig. 21b-c, p=2.2 x 10^{-3} veh. vs. 10 μm ento.) (Ref. II.).

During our in vitro experiments, we also tested the effects of another Syk-selective inhibitor, lanraplenib on human granulocytes. Lanraplenib dose-dependently reduced the superoxide release and cell spreading of neutrophils (Fig. 21d-e, p=0.019 veh. vs. 10 μm lanra., p=0.024 veh. vs. 0.1 μm lanra., respectively) (Ref. II.).

Taken together, selective Syk-inhibition decreased the effector cell responses of immune complex-activated human neutrophils (Ref. II.). These results with the in vivo observations can indicate that Syk could be a potential therapeutic target in the treatment of autoimmune inflammation in the future, and the Syk-selective entospletinib or lanraplenib could be potential therapeutic options (Ref. I. and Ref. II.).

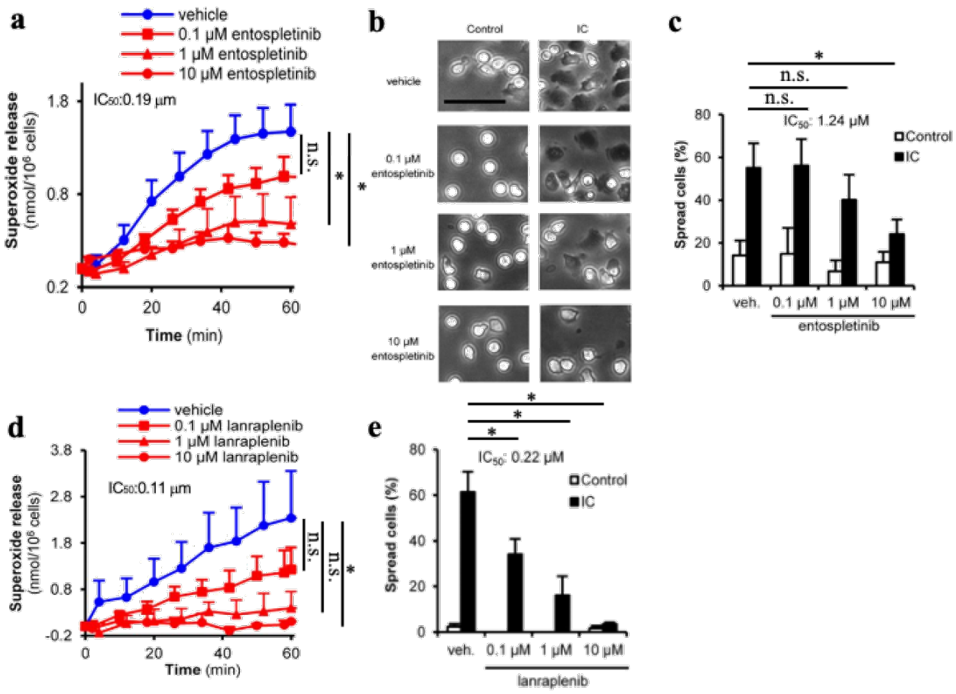

Figure 21: The Syk-selective inhibition effectively decreased the cell responses of human neutrophils

*The selective Syk-inhibitor entospletinib and lanraplenib nicely decreased the superoxide release (a,d) and cell spreading (b-c, e) of human neutrophils. Curves (a,c-d) and diagrams (c-e) show mean and SEM from 3-6 independent experiments (control data were subtracted on panel a-d). Representative pictures (b) from three independent experiments are shown. IC, immune complex; veh., vehicle; *p<0.05, Scale bar: 50 μm. (Ref. II.).*

5. Discussion

Autoimmune diseases affect approximately 5 % of the population (126). Rheumatoid arthritis is a systemic autoimmune disorder, which is associated with joint inflammation, pain and in some cases, it can cause mobility impairment (14, 127-129). In a proportion (up to 5-20 %) of RA patients, remission cannot be reached by the available therapies, so there is a need to understand the pathogenesis in more details and to find new therapeutic targets and drug candidates in the treatment of the disease.

In seropositive rheumatoid arthritis, immune complexes are formed by autoantigens and autoantibodies (130, 131). Immune complexes are thought to play a significant role in certain autoantibody-mediated inflammatory processes, such as in K/BxN serum transfer arthritis, which partially models rheumatoid arthritis. In the experimental autoantibody-mediated K/BxN serum transfer arthritis model, the accumulation of glucose-6-phosphate isomerase and its specific anti-G6PI antibodies are typically observed in the joint cartilage area (132). In line with this phenomenon, in the pathogenesis of another immune complex-mediated autoimmune disease, in epidermolysis bullosa acquisita, immune complexes are formed by type VII collagen and anti-type VII collagen antibodies in the dermal-epidermal junction of the skin (133). Immune complexes are able to bind to Fcγ receptors and they can activate the downstream signalling pathways (84, 115). It has been shown that deficiency of Fc receptors γ-chain resulted in a protection from K/BxN serum transfer arthritis (102, 106, 107). The lack of FcγRIII partially protected mice from experimental joint inflammation, while FcγRI did not play an important role in the disease (102).

β2-integrins are important compartment of the autoimmune processes (134). It has been shown that the absence of CD18 resulted in a protection from the development of K/BxN serum transfer arthritis (135). In this process the expression of LFA-1 was crucial. The treatment with anti-CD11a (anti-LFA-1) antibodies inhibited the development of arthritis in mice (135). In an additional experiment, Gfi-1$^{-/-}$ mice were irradiated and then transplanted with LFA-1-deficient bone marrow cells (110). After repopulation, the LFA-1-deficient mice became protected against the development of arthritis compared to the wild type animals, further strengthening the fact that LFA-1 expression on the surface of neutrophils is essential for the development of autoimmune arthritis (110). However, the

absence of Mac-1 on neutrophils did not reduce the severity of K/BxN serum transfer arthritis, but it was proved to be important in collagen-induced arthritis (102).

A shared point between the FcγR and β2-integrin is that both of them can use the Syk tyrosine-kinase for their signalling cascade. It has been shown that Syk has a crucial role in the development of autoantibody-induced experimental arthritis (Ref. I.). The absence of Syk from the hematopoietic compartment resulted in a total protection from experimental K/BxN serum transfer arthritis, in a model, where the neutrophils, platelets and mast cells have critical roles (113). A common element in the signalling of these cells is the Syk tyrosine-kinase, therefore it was an interesting question how Syk in these cells contributed to the progression of autoimmune arthritis. Our workgroup and others have shown that the cell-specific deletion of Syk from neutrophils resulted in a protection from the development of autoantibody-induced arthritis, while the absence of Syk in platelets and mast cells did not reduce the severity of joint inflammation (99, 134). This phenomenon may highlight that the expression of Syk in neutrophils is important in the development of autoantibody-induced arthritis (134).

These data with further observations raised the possibility that Syk could be an important component of autoimmune arthritis and it can be a potential promising drug target in the treatment of human autoimmune diseases. During the past decades, fostamatinib has been tested as a Syk inhibitor in experimental arthritis (136). Fostamatinib seemed to be effective in arthritic mice, and also in phase 2 clinical trials in patients with RA (136, 137). However, fostamatinib failed in phase 3 clinical studies as it showed only a moderate effect. Further experiments showed that fostamatinib is not a Syk-selective inhibitor, but rather a non-selective (general) tyrosine-kinase blocker (114). Entospletinib is a second generation, Syk-selective inhibitor and it has a tolerable safety profile according to haematological clinical trials in patients with chronic lymphoid leukaemia (114, 138). During our study, our first aim was to test how pharmacological inhibition of Syk affects the development of autoantibody-induced experimental arthritis (Ref. I.).

In our experiments, the Syk-selective entospletinib or vehicle was administered orally twice a day. The severity of joint inflammation was followed by clinical scoring and ankle thickness measurements (Ref. I.). The lower dose of entospletinib could moderately decrease the severity of joint inflammation as shown on the clinical score and

ankle thickness measurement curves (Figure 11), while entospletinib-treatment did not decrease the cell numbers in the bone marrow, in the peripheral blood or in the synovial area under non-inflammatory conditions (Figure 10). When we administered a higher-dose of entospletinib, it massively reduced the severity of joint inflammation (Figure 12) (Ref. I.). We observed that entospletinib-treatment did not cause visible health problems and did not decrease the weight of mice compared to the vehicle-treated group (Figure 12). We can conclude that the Syk-selective entospletinib could dose-dependently reduce the severity of autoantibody-induced experimental arthritis without decreasing the bone marrow, peripheral and synovial cell numbers under non-inflammatory conditions (Ref. I.).

As we have previously mentioned, in the pathogenesis of RA, many cell types are involved (like neutrophils, macrophages, synovial fibroblasts etc.) (17, 139). In our experiments, we tested how entospletinib affected leukocyte infiltration to the joints. During our flow cytometric analysis, we saw that entospletinib-treatment reduced the local neutrophil numbers at both doses of the inhibitor upon arthritis induction, while the local macrophage and synovial fibroblast numbers did not decrease (Figure 13) (Ref. I.). Synovial fibroblasts can take part in antigen presentation by the expression of MHC Class II. Under inflammatory conditions, the MHC Class II expression can be increased. In our experiments, we followed the activation status of synovial fibroblasts by labelling with anti-MHC Class II antibodies. We saw that entospletinib-treatment significantly decreased the MHC Class II expression in mice treated with a higher dose of entospletinib (Figure 14) (Ref. I.). Next, we wanted to test the intracellular tyrosine phosphorylation of synovial fibroblasts. After permeabilization and fixation steps, we measured it by flow cytometry. The selective Syk inhibition by entospletinib did not reduce the intracellular tyrosine phosphorylation of the synovial fibroblasts compared to the vehicle-treated group (Figure 14) (Ref. I.). To sum it up, entospletinib did not decrease the numbers of macrophage and synovial fibroblasts, while the local neutrophil numbers were significantly reduced after arthritis induction. These results suggest that mainly the neutrophil-dependent mechanisms could stand behind the macroscopic phenotype. Entospletinib is unlikely to affect neutrophil cell migration, as it has been shown that the absence of Syk from neutrophils did not influence the migratory capacity under in vivo and in vitro conditions (74, 115). We think that entospletinib exerts its inhibitory effect,

when neutrophils arrive to the site of inflammation, and are activated by immune complexes or through integrins. We believe that tissue inflammation is amplified through Fc receptor and integrin signalling cascades by a positive feedback loop, which are generated by neutrophils (Ref. I.).

Another effector cell type in the pathogenesis of rheumatoid arthritis is the B cell, and it can be interesting how entospletinib affects these lymphocytes (Ref. I.). In our studies, we used a passive immunization mouse model where B cells are dispensable. We isolated B cells from the bone marrow and we measured the basal intracellular tyrosine phosphorylation in the presence of entospletinib. We saw that the basal intracellular tyrosine phosphorylation was reduced in the entospletinib-treated samples (data not shown, Ref. I.). This observation supports that B cells can be inhibited by entospletinib and it will be interesting to test it in another, active immunization-based arthritis model. In the pathogenesis of autoimmune arthritis, several cytokines and chemokines have a critical role (Ref. I.). MIP1-α is an important pro-inflammatory mediator, which can increase the accumulation of effector cells under inflammatory conditions (140). Furthermore, MIP-2 can be a crucial player in early inflammatory responses, while IL-1β can amplify the inflammation (141, 142). In our study, we measured the local MIP1-α (CCL3), MIP-2 (CXCL2) and IL-1β levels by ELISA. We saw that entospletinib-treatment significantly decreased the levels of pro-inflammatory mediators compared to the vehicle-treated animals (Figure 15) (Ref. I.).

Next, we in vitro validated our in vivo observations, so we followed the FcγR- and β2-integrin-mediated neutrophil cell responses in the presence of entospletinib. We found that entospletinib did not reduce the expression of the maturation marker Ly-6G, FcγRII/III and FcγRIV or integrin α and β chains (Figure 16 and Figure 19) (Ref. I.). Meanwhile, immune complex (Figure 17 and Figure 18) or integrin-triggered (Figure 20) neutrophil superoxide release, cell spreading and cytokine production were dose-dependently reduced by entospletinib (Ref. I. and Ref. II.). We think that entospletinib achieves the inhibitory effect when neutrophils are activated through their FcγRs or β2-integrins at the site of inflammation and the initiate of a positive feedback loop. We have to mention that during our study, the mouse half-maximal serum (IC_{50}) levels of entospletinib were lower, than the maximum serum level in entospletinib-treated human individuals (114, 138). That means that clinically relevant entospletinib doses were used

in our experiments (143, 144). In the experiment of Poe and colleagues, where the mice were administered 80 and 240 mg entospletinib per day, the plasma concentrations reached 1.33 μM and 3.48 μM, which are above our in vitro entospletinib IC_{50} values (144).

To strengthen the translational aspects of our study, we tested the effect of entospletinib and lanraplenib on human granulocytes (Ref. II.). We found that entospletinib and lanraplenib were able to decrease the superoxide production and cell spreading of human neutrophils in a dose-dependent manner (Figure 21) (Ref. II.). These results with our in vivo observations indicate that Syk could be a therapeutic target in the treatment of autoimmune inflammation and both Syk-selective inhibitors (entospletinib and lanraplenib) could be potential therapeutic options.

6. Conclusions

Our new findings are the followings:

1) The oral administration of the Syk-selective inhibitor entospletinib effectively reduced the severity of a neutrophil-, Fc receptor- and integrin-dependent autoantibody-mediated experimental arthritis.

2) We also noted that entospletinib effectively reduced the recruitment of neutrophils to the joints and also lowered the levels of pro-inflammatory mediators at the site of inflammation upon arthritis induction.

3) We also found that under in vitro conditions, the selective Syk inhibition dose-dependently decreased the investigated cell responses of isolated mouse neutrophils, while the maturation, the Fcγ receptor and the β2-integrin expression was not altered.

4) Selective Syk-inhibition by entospletinib and lanraplenib decreased the immune complex-mediated human neutrophil superoxide release and cell spreading.

7. Summary

Syk is a non-receptor tyrosine-kinase, which is an important component of Fcγ receptor or β2-integrin signalling. It has been shown that the absence of Syk from the hematopoietic compartment resulted in a total protection from the development of experimental arthritis. This result with further observations raised the possibility to test the effect of Syk-selective inhibition in autoantibody-induced experimental arthritis.

In our experiments, we investigated the effect of a Syk-selective inhibitor (namely entospletinib) in autoantibody-induced experimental arthritis and in in vitro immune-complex and integrin-mediated cell responses of mouse and human neutrophils.

Experimental arthritis was induced by intraperitoneal injection of K/BxN serum. Entospletinib or vehicle were administered orally twice a day. Arthritis was followed by clinical scoring and ankle thickness measurements. The local cytokine levels were detected by ELISA, while the local cell recruitment, synovial fibroblast numbers and the activation status were detected by flow cytometry. In our in vitro experiments, immune-complex-or integrin-triggered neutrophil superoxide release was measured by a cytochrome c-reduction assay, cell spreading was detected by phase contrast microscopy and cytokine production was followed by ELISA.

The oral administration of entospletinib decreased the severity of experimental arthritis. In line with this, the number of synovial neutrophils, the MHC II expression of synovial fibroblasts and the synovial cytokine levels were decreased in the entospletinib-treated group. In line with these findings, entospletinib reduced the immune complex- and integrin-triggered mouse neutrophil cell responses in a dose-dependent manner. Furthermore, entospletinib and lanraplenib dose-dependently decreased the investigated human neutrophil effector responses.

The Syk-selective inhibitor entospletinib could reduce joint inflammation in experimental arthritis, while the tested inhibitors effectively reduced the in vitro investigated neutrophil effector cell responses. Our data raise the possibility that entospletinib (or lanraplenib) could be good drug candidates in the treatment of human autoimmune arthritis.

8. References

1. Desai MK, Brinton RD. Autoimmune Disease in Women: Endocrine Transition and Risk Across the Lifespan. Front Endocrinol (Lausanne). 2019;10:265.

2. Davidson A, Diamond B. Autoimmune diseases. N Engl J Med. 2001;345(5):340-50.

3. Siegel RJ, Bridges SL, Jr., Ahmed S. HLA-C: An Accomplice in Rheumatic Diseases. ACR Open Rheumatol. 2019;1(9):571-9.

4. Pisetsky DS. Pathogenesis of autoimmune disease. Nat Rev Nephrol. 2023;19(8):509-24.

5. Mazzone R, Zwergel C, Artico M, Taurone S, Ralli M, Greco A, et al. The emerging role of epigenetics in human autoimmune disorders. Clin Epigenetics. 2019;11(1):34.

6. Ying D, Schmajuk G, Trupin L, Blanc PD. Inorganic Dust Exposure During Military Service as a Predictor of Rheumatoid Arthritis and Other Autoimmune Conditions. ACR Open Rheumatol. 2021;3(7):466-74.

7. Xiang Y, Zhang M, Jiang D, Su Q, Shi J. The role of inflammation in autoimmune disease: a therapeutic target. Front Immunol. 2023;14:1267091.

8. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2018;9(6):7204-18.

9. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. Bone Res. 2018;6:15.

10. Fava A, Petri M. Systemic lupus erythematosus: Diagnosis and clinical management. J Autoimmun. 2019;96:1-13.

11. Bukiri H, Volkmann ER. Current advances in the treatment of systemic sclerosis. Curr Opin Pharmacol. 2022;64:102211.

12. Szilveszter KP, Vikár S, Horváth AI, Helyes Z, Sárdy M, Mócsai A. Phospholipase Cγ2 Is Essential for Experimental Models of Epidermolysis Bullosa Acquisita. J Invest Dermatol. 2022;142(4):1114-25.

13. Kridin K, Kneiber D, Kowalski EH, Valdebran M, Amber KT. Epidermolysis bullosa acquisita: A comprehensive review. Autoimmun Rev. 2019;18(8):786-95.

14. Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, et al. Rheumatoid arthritis. Nat Rev Dis Primers. 2018;4:18001.

15. Di Matteo A, Bathon JM, Emery P. Rheumatoid arthritis. Lancet. 2023;402(10416):2019-33.

16.Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet. 2016;388(10055):2023-38.
17. Németh T, Nagy G, Pap T. Synovial fibroblasts as potential drug targets in rheumatoid arthritis, where do we stand and where shall we go? Ann Rheum Dis. 2022;81(8):1055-64.
18. Fresneda Alarcon M, McLaren Z, Wright HL. Neutrophils in the Pathogenesis of Rheumatoid Arthritis and Systemic Lupus Erythematosus: Same Foe Different M.O. Front Immunol. 2021;12:649693.
19. Zhou S, Lu H, Xiong M. Identifying Immune Cell Infiltration and Effective Diagnostic Biomarkers in Rheumatoid Arthritis by Bioinformatics Analysis. Front Immunol. 2021;12:726747.
20. Cecchi I, Arias de la Rosa I, Menegatti E, Roccatello D, Collantes-Estevez E, Lopez-Pedrera C, et al. Neutrophils: Novel key players in Rheumatoid Arthritis. Current and future therapeutic targets. Autoimmun Rev. 2018;17(11):1138-49.
21.Yokota K. Osteoclast differentiation in rheumatoid arthritis. Immunol Med. 2024;47(1):6-11.
22. Figus FA, Piga M, Azzolin I, McConnell R, Iagnocco A. Rheumatoid arthritis: Extra-articular manifestations and comorbidities. Autoimmun Rev. 2021;20(4):102776.
23. Das S, Padhan P. An Overview of the Extraarticular Involvement in Rheumatoid Arthritis and its Management. J Pharmacol Pharmacother. 2017;8(3):81-6.
24. Bernardinello N, Zen M, Castelli G, Cocconcelli E, Balestro E, Borie R, et al. Navigating interstitial lung disease associated with rheumatoid arthritis (RA-ILD): from genetics to clinical landscape. Front Med (Lausanne). 2025;12:1542400.
25. Süß P, Rothe T, Hoffmann A, Schlachetzki JCM, Winkler J. The Joint-Brain Axis: Insights From Rheumatoid Arthritis on the Crosstalk Between Chronic Peripheral Inflammation and the Brain. Front Immunol. 2020;11:612104.
26. Fan S, Zhao J, Hou B, Liu M, Niu J, Zhou Y, et al. Rheumatoid meningitis: a rare neurological complication of rheumatoid arthritis. Front Immunol. 2023;14:1065650.
27. Jiang Z, Chen L, Liu A, Qi J, Wang J, Li Y, et al. Rheumatoid arthritis and the risk of chronic kidney diseases: a Mendelian randomization study. Front Med (Lausanne). 2024;11:1360026.

28. Nagy G, Roodenrijs NMT, Welsing PM, Kedves M, Hamar A, van der Goes MC, et al. EULAR definition of difficult-to-treat rheumatoid arthritis. Ann Rheum Dis. 2021;80(1):31-5.
29.Smolen JS, Landewe RBM, Bijlsma JWJ, Burmester GR, Dougados M, Kerschbaumer A, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. Ann Rheum Dis. 2020;79(6):685-99.
30. Nagy G, Roodenrijs NMT, Welsing PMJ, Kedves M, Hamar A, van der Goes MC, et al. EULAR points to consider for the management of difficult-to-treat rheumatoid arthritis. Ann Rheum Dis. 2022;81(1):20-33.
31. Smolen JS, Landewe RBM, Bergstra SA, Kerschbaumer A, Sepriano A, Aletaha D, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2022 update. Ann Rheum Dis. 2023;82(1):3-18.
32. Balogh L, Oláh K, Sánta S, Majerhoffer N, Németh T. Novel and potential future therapeutic options in systemic autoimmune diseases. Front Immunol. 2024;15:1249500.
33. Caporali R, Taylor PC, Aletaha D, Sanmarti R, Takeuchi T, Mo D, et al. Efficacy of baricitinib in patients with moderate-to-severe rheumatoid arthritis up to 6.5 years of treatment: results of a long-term study. Rheumatology (Oxford). 2024;63(10):2799-809.
34. Dhillon S. Tofacitinib: A Review in Rheumatoid Arthritis. Drugs. 2017;77(18):1987-2001.
35. Kaneko Y. Efficacy and safety of peficitinib in rheumatoid arthritis. Mod Rheumatol. 2020;30(5):773-8.
36. Kucharz EJ, Stajszczyk M, Kotulska-Kucharz A, Batko B, Brzosko M, Jeka S, et al. Tofacitinib in the treatment of patients with rheumatoid arthritis: position statement of experts of the Polish Society for Rheumatology. Reumatologia. 2018;56(4):203-11.
37. Sanmarti R, Corominas H. Upadacitinib for Patients with Rheumatoid Arthritis: A Comprehensive Review. J Clin Med. 2023;12(5).
38. Urits I, Israel J, Hakobyan H, Yusin G, Lassiter G, Fackler N, et al. Baricitinib for the treatment of rheumatoid arthritis. Reumatologia. 2020;58(6):407-15.

39. Hoisnard L, Lebrun-Vignes B, Maury S, Mahevas M, El Karoui K, Roy L, et al. Adverse events associated with JAK inhibitors in 126,815 reports from the WHO pharmacovigilance database. Sci Rep. 2022;12(1):7140.
40. Kurkó J, Besenyei T, Laki J, Glant TT, Mikecz K, Szekanecz Z. Genetics of rheumatoid arthritis - a comprehensive review. Clin Rev Allergy Immunol. 2013;45(2):170-9.
41. Deane KD, Demoruelle MK, Kelmenson LB, Kuhn KA, Norris JM, Holers VM. Genetic and environmental risk factors for rheumatoid arthritis. Best Pract Res Clin Rheumatol. 2017;31(1):3-18.
42. Finckh A, Gilbert B, Hodkinson B, Bae SC, Thomas R, Deane KD, et al. Global epidemiology of rheumatoid arthritis. Nat Rev Rheumatol. 2022;18(10):591-602.
43. van Drongelen V, Holoshitz J. Human Leukocyte Antigen-Disease Associations in Rheumatoid Arthritis. Rheum Dis Clin North Am. 2017;43(3):363-76.
44. Mustelin T, Bottini N, Stanford SM. The Contribution of PTPN22 to Rheumatic Disease. Arthritis Rheumatol. 2019;71(4):486-95.
45. Knol EF, Gilles S. Allergy: Type I, II, III, and IV. Handb Exp Pharmacol. 2022;268:31-41.
46. Bissenova S, Ellis D, Mathieu C, Gysemans C. Neutrophils in autoimmunity: when the hero becomes the villain. Clin Exp Immunol. 2022;210(2):128-40.
47. Ingegnoli F, Castelli R, Gualtierotti R. Rheumatoid factors: clinical applications. Dis Markers. 2013;35(6):727-34.
48. Takeshita M, Suzuki K, Kondo Y, Morita R, Okuzono Y, Koga K, et al. Multi-dimensional analysis identified rheumatoid arthritis-driving pathway in human T cell. Ann Rheum Dis. 2019;78(10):1346-56.
49. Machado CRL, Firestein GS. Fibroblast multiplicity in RA: a synovial state of affairs. Nat Rev Rheumatol. 2023;19(10):609-10.
50. Mizoguchi F, Slowikowski K, Wei K, Marshall JL, Rao DA, Chang SK, et al. Functionally distinct disease-associated fibroblast subsets in rheumatoid arthritis. Nat Commun. 2018;9(1):789.
51. Qian H, Deng C, Chen S, Zhang X, He Y, Lan J, et al. Targeting pathogenic fibroblast-like synoviocyte subsets in rheumatoid arthritis. Arthritis Res Ther. 2024;26(1):103.

52. Niu Q, Gao J, Wang L, Liu J, Zhang L. Regulation of differentiation and generation of osteoclasts in rheumatoid arthritis. Front Immunol. 2022;13:1034050.
53. Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. Rheumatology (Oxford). 2010;49(9):1618-31.
54. Manz MG, Boettcher S. Emergency granulopoiesis. Nat Rev Immunol. 2014;14(5):302-14.
55. Németh T, Sperandio M, Mócsai A. Neutrophils as emerging therapeutic targets. Nat Rev Drug Discov. 2020;19(4):253-75.
56. Ganesh K, Joshi MB. Neutrophil sub-types in maintaining immune homeostasis during steady state, infections and sterile inflammation. Inflamm Res. 2023;72(6):1175-92.
57. Lawrence SM, Corriden R, Nizet V. The Ontogeny of a Neutrophil: Mechanisms of Granulopoiesis and Homeostasis. Microbiol Mol Biol Rev. 2018;82(1).
58. Strydom N, Rankin SM. Regulation of circulating neutrophil numbers under homeostasis and in disease. J Innate Immun. 2013;5(4):304-14.
59. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Frontiers in Physiology. 2018;9.
60. Malengier-Devlies B, Metzemaekers M, Wouters C, Proost P, Matthys P. Neutrophil Homeostasis and Emergency Granulopoiesis: The Example of Systemic Juvenile Idiopathic Arthritis. Front Immunol. 2021;12:766620.
61. Burn GL, Foti A, Marsman G, Patel DF, Zychlinsky A. The Neutrophil. Immunity. 2021;54(7):1377-91.
62. Wang Y, Jonsson F. Expression, Role, and Regulation of Neutrophil Fcγ Receptors. Front Immunol. 2019;10:1958.
63. Futosi K, Fodor S, Mócsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. Int Immunopharmacol. 2013;17(3):638-50.
64. Mócsai A, Walzog B, Lowell CA. Intracellular signalling during neutrophil recruitment. Cardiovasc Res. 2015;107(3):373-85.
65. Filippi MD. Neutrophil transendothelial migration: updates and new perspectives. Blood. 2019;133(20):2149-58.

66. Kruger P, Saffarzadeh M, Weber AN, Rieber N, Radsak M, von Bernuth H, et al. Neutrophils: Between host defence, immune modulation, and tissue injury. PLoS Pathog. 2015;11(3):e1004651.
67. Parker HA, Forrester L, Kaldor CD, Dickerhof N, Hampton MB. Antimicrobial Activity of Neutrophils Against Mycobacteria. Front Immunol. 2021;12:782495.
68. Korkmaz B, Horwitz MS, Jenne DE, Gauthier F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev. 2010;62(4):726-59.
69. Wang H, Kim SJ, Lei Y, Wang S, Wang H, Huang H, et al. Neutrophil extracellular traps in homeostasis and disease. Signal Transduct Target Ther. 2024;9(1):235.
70. Rosales C, Uribe-Querol E. Phagocytosis: A Fundamental Process in Immunity. Biomed Res Int. 2017;2017:9042851.
71. Moffat A, Gwyer Findlay E. Evidence for antigen presentation by human neutrophils. Blood. 2024;143(24):2455-63.
72. Polak D, Bohle B. Neutrophils-typical atypical antigen presenting cells? Immunol Lett. 2022;247:52-8.
73. Dahlgren C, Forsman H, Sundqvist M, Bjorkman L, Martensson J. Signaling by neutrophil G protein-coupled receptors that regulate the release of superoxide anions. J Leukoc Biol. 2024;116(6):1334-51.
74. Mócsai A, Zhou M, Meng F, Tybulewicz VL, Lowell CA. Syk is required for integrin signaling in neutrophils. Immunity. 2002;16(4):547-58.
75. Daeron M. Fc receptors as adaptive immunoreceptors. Curr Top Microbiol Immunol. 2014;382:131-64.
76. Ben Mkaddem S, Benhamou M, Monteiro RC. Understanding Fc Receptor Involvement in Inflammatory Diseases: From Mechanisms to New Therapeutic Tools. Front Immunol. 2019;10:811.
77. Junker F, Gordon J, Qureshi O. Fcγ Receptors and Their Role in Antigen Uptake, Presentation, and T Cell Activation. Front Immunol. 2020;11:1393.
78. Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. Nat Rev Immunol. 2008;8(1):34-47.

79. Bournazos S, Woof JM, Hart SP, Dransfield I. Functional and clinical consequences of Fc receptor polymorphic and copy number variants. Clin Exp Immunol. 2009;157(2):244-54.
80. Smith KGC, Clatworthy MR. FcγRIIB in autoimmunity and infection: evolutionary and therapeutic implications. Nature Reviews Immunology. 2010;10(5):328-43.
81. Nagelkerke SQ, Schmidt DE, de Haas M, Kuijpers TW. Genetic Variation in Low-To-Medium-Affinity Fcγ Receptors: Functional Consequences, Disease Associations, and Opportunities for Personalized Medicine. Front Immunol. 2019;10:2237.
82. Sepúlveda-Delgado J, Llorente L, Hernández-Doño S. A Comprehensive Review of Fcγ Receptors and Their Role in Systemic Lupus Erythematosus. International Journal of Molecular Sciences. 2025;26(5).
83. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcγRIV: a novel FcR with distinct IgG subclass specificity. Immunity. 2005;23(1):41-51.
84. Mócsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol. 2010;10(6):387-402.
85. Sun L, Guo S, Xie Y, Yao Y. The characteristics and the multiple functions of integrin β1 in human cancers. J Transl Med. 2023;21(1):787.
86. Mezu-Ndubuisi OJ, Maheshwari A. The role of integrins in inflammation and angiogenesis. Pediatr Res. 2021;89(7):1619-26.
87. Bednarczyk M, Stege H, Grabbe S, Bros M. β2 Integrins-Multi-Functional Leukocyte Receptors in Health and Disease. Int J Mol Sci. 2020;21(4).
88. Hu T, Zhang Z, Deng C, Ma X, Liu X. Effects of β2 Integrins on Osteoclasts, Macrophages, Chondrocytes, and Synovial Fibroblasts in Osteoarthritis. Biomolecules. 2022;12(11).
89. Fagerholm SC, Guenther C, Llort Asens M, Savinko T, Uotila LM. β2-Integrins and Interacting Proteins in Leukocyte Trafficking, Immune Suppression, and Immunodeficiency Disease. Front Immunol. 2019;10:254.
90. Sun H, Zhi K, Hu L, Fan Z. The Activation and Regulation of β2 Integrins in Phagocytes and Phagocytosis. Front Immunol. 2021;12:633639.
91. Schittenhelm L, Hilkens CM, Morrison VL. β2 Integrins As Regulators of Dendritic Cell, Monocyte, and Macrophage Function. Front Immunol. 2017;8:1866.

92. Solouki S, August A, Huang W. Non-receptor tyrosine kinase signaling in autoimmunity and therapeutic implications. Pharmacol Ther. 2019;201:39-50.
93. Beyer C, Distler JH. Tyrosine kinase signaling in fibrotic disorders: Translation of basic research to human disease. Biochim Biophys Acta. 2013;1832(7):897-904.
94. Esteban-Villarrubia J, Soto-Castillo JJ, Pozas J, San Roman-Gil M, Orejana-Martin I, Torres-Jimenez J, et al. Tyrosine Kinase Receptors in Oncology. Int J Mol Sci. 2020;21(22).
95. D'Aura Swanson C, Paniagua RT, Lindstrom TM, Robinson WH. Tyrosine kinases as targets for the treatment of rheumatoid arthritis. Nat Rev Rheumatol. 2009;5(6):317-24.
96. Paul MK, Mukhopadhyay AK. Tyrosine kinase - Role and significance in Cancer. Int J Med Sci. 2004;1(2):101-15.
97. Gocek E, Moulas AN, Studzinski GP. Non-receptor protein tyrosine kinases signaling pathways in normal and cancer cells. Crit Rev Clin Lab Sci. 2014;51(3):125-37.
98. Eshaq AM, Flanagan TW, Hassan SY, Al Asheikh SA, Al-Amoudi WA, Santourlidis S, et al. Non-Receptor Tyrosine Kinases: Their Structure and Mechanistic Role in Tumor Progression and Resistance. Cancers (Basel). 2024;16(15).
99. Németh T, Futosi K, Szilveszter K, Vilinovszki O, Kiss-Papai L, Mócsai A. Lineage-Specific Analysis of Syk Function in Autoantibody-Induced Arthritis. Front Immunol. 2018;9:555.
100. Siveen KS, Prabhu KS, Achkar IW, Kuttikrishnan S, Shyam S, Khan AQ, et al. Role of Non Receptor Tyrosine Kinases in Hematological Malignances and its Targeting by Natural Products. Mol Cancer. 2018;17(1):31.
101. Herrero-Cervera A, Soehnlein O, Kenne E. Neutrophils in chronic inflammatory diseases. Cell Mol Immunol. 2022;19(2):177-91.
102. Németh T, Mócsai A. The role of neutrophils in autoimmune diseases. Immunol Lett. 2012;143(1):9-19.
103. Zhang L, Yuan Y, Xu Q, Jiang Z, Chu CQ. Contribution of neutrophils in the pathogenesis of rheumatoid arthritis. J Biomed Res. 2019;34(2):86-93.
104. Wright HL, Lyon M, Chapman EA, Moots RJ, Edwards SW. Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines,

Reactive Oxygen Species, and Neutrophil Extracellular Traps. Front Immunol. 2020;11:584116.
105. Chen W, Wang Q, Ke Y, Lin J. Neutrophil Function in an Inflammatory Milieu of Rheumatoid Arthritis. J Immunol Res. 2018;2018:8549329.
106. Németh T, Futosi K, Szabó M, Aradi P, Saito T, Mócsai A, et al. Importance of Fc Receptor γ-Chain ITAM Tyrosines in Neutrophil Activation and in vivo Autoimmune Arthritis. Front Immunol. 2019;10:252.
107. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, et al. Arthritis critically dependent on innate immune system players. Immunity. 2002;16(2):157-68.
108. Magnusson SE, Wennerberg E, Matt P, Lindqvist U, Kleinau S. Dysregulated Fc receptor function in active rheumatoid arthritis. Immunol Lett. 2014;162(1 Pt A):200-6.
109. Nimmerjahn F, Lux A, Albert H, Woigk M, Lehmann C, Dudziak D, et al. FcγRIV deletion reveals its central role for IgG2a and IgG2b activity in vivo. Proc Natl Acad Sci U S A. 2010;107(45):19396-401.
110. Monach PA, Nigrovic PA, Chen M, Hock H, Lee DM, Benoist C, et al. Neutrophils in a mouse model of autoantibody-mediated arthritis: critical producers of Fc receptor γ, the receptor for C5a, and lymphocyte function-associated antigen 1. Arthritis Rheum. 2010;62(3):753-64.
111. Lowin T, Straub RH. Integrins and their ligands in rheumatoid arthritis. Arthritis Res Ther. 2011;13(5):244.
112. Mitroulis I, Alexaki VI, Kourtzelis I, Ziogas A, Hajishengallis G, Chavakis T. Leukocyte integrins: role in leukocyte recruitment and as therapeutic targets in inflammatory disease. Pharmacol Ther. 2015;147:123-35.
113. Jakus Z, Simon E, Balázs B, Mócsai A. Genetic deficiency of Syk protects mice from autoantibody-induced arthritis. Arthritis Rheum. 2010;62(7):1899-910.
114. Sharman J, Hawkins M, Kolibaba K, Boxer M, Klein L, Wu M, et al. An open-label phase 2 trial of entospletinib (GS-9973), a selective spleen tyrosine kinase inhibitor, in chronic lymphocytic leukemia. Blood. 2015;125(15):2336-43.
115. Németh T, Virtic O, Sitaru C, Mócsai A. The Syk Tyrosine Kinase Is Required for Skin Inflammation in an In Vivo Mouse Model of Epidermolysis Bullosa Acquisita. J Invest Dermatol. 2017;137(10):2131-9.

116. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity. 1999;10(4):451-61.
117. Kovács M, Németh T, Jakus Z, Sitaru C, Simon E, Futosi K, et al. The Src family kinases Hck, Fgr, and Lyn are critical for the generation of the in vivo inflammatory environment without a direct role in leukocyte recruitment. J Exp Med. 2014;211(10):1993-2011.
118. Wipke BT, Allen PM. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol. 2001;167(3):1601-8.
119. Christensen AD, Haase C, Cook AD, Hamilton JA. K/BxN Serum-Transfer Arthritis as a Model for Human Inflammatory Arthritis. Front Immunol. 2016;7:213.
120. Jakus Z, Simon E, Frommhold D, Sperandio M, Mócsai A. Critical role of phospholipase Cγ2 in integrin and Fc receptor-mediated neutrophil functions and the effector phase of autoimmune arthritis. J Exp Med. 2009;206(3):577-93.
121. Németh T, Futosi K, Sitaru C, Ruland J, Mócsai A. Neutrophil-specific deletion of the CARD9 gene expression regulator suppresses autoantibody-induced inflammation in vivo. Nat Commun. 2016;7:11004.
122. Mócsai A, Zhang H, Jakus Z, Kitaura J, Kawakami T, Lowell CA. G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells. Blood. 2003;101(10):4155-63.
123. Futosi K, Németh T, Pick R, Vantus T, Walzog B, Mócsai A. Dasatinib inhibits proinflammatory functions of mature human neutrophils. Blood. 2012;119(21):4981-91.
124. Jakus Z, Németh T, Verbeek JS, Mócsai A. Critical but overlapping role of FcγRIII and FcγRIV in activation of murine neutrophils by immobilized immune complexes. J Immunol. 2008;180(1):618-29.
125. Németh T, Futosi K, Hably C, Brouns MR, Jakob SM, Kovács M, et al. Neutrophil functions and autoimmune arthritis in the absence of p190RhoGAP: generation and analysis of a novel null mutation in mice. J Immunol. 2010;185(5):3064-75.
126. Amador-Patarroyo MJ, Rodriguez-Rodriguez A, Montoya-Ortiz G. How does age at onset influence the outcome of autoimmune diseases? Autoimmune Dis. 2012;2012:251730.

127. Gao Y, Zhang Y, Liu X. Rheumatoid arthritis: pathogenesis and therapeutic advances. MedComm (2020). 2024;5(3):e509.
128. Bullock J, Rizvi SAA, Saleh AM, Ahmed SS, Do DP, Ansari RA, et al. Rheumatoid Arthritis: A Brief Overview of the Treatment. Med Princ Pract. 2018;27(6):501-7.
129. Radu AF, Bungau SG. Management of Rheumatoid Arthritis: An Overview. Cells. 2021;10(11).
130. Biswas S, Sharma S, Saroha A, Bhakuni DS, Malhotra R, Zahur M, et al. Identification of novel autoantigen in the synovial fluid of rheumatoid arthritis patients using an immunoproteomics approach. PLoS One. 2013;8(2):e56246.
131. Derksen V, Huizinga TWJ, van der Woude D. The role of autoantibodies in the pathophysiology of rheumatoid arthritis. Semin Immunopathol. 2017;39(4):437-46.
132. Matsumoto I, Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, et al. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. Nat Immunol. 2002;3(4):360-5.
133. Sitaru C, Kromminga A, Hashimoto T, Brocker EB, Zillikens D. Autoantibodies to type VII collagen mediate Fcγ-dependent neutrophil activation and induce dermal-epidermal separation in cryosections of human skin. Am J Pathol. 2002;161(1):301-11.
134. Elliott ER, Van Ziffle JA, Scapini P, Sullivan BM, Locksley RM, Lowell CA. Deletion of Syk in neutrophils prevents immune complex arthritis. J Immunol. 2011;187(8):4319-30.
135. Watts GM, Beurskens FJ, Martin-Padura I, Ballantyne CM, Klickstein LB, Brenner MB, et al. Manifestations of inflammatory arthritis are critically dependent on LFA-1. J Immunol. 2005;174(6):3668-75.
136. Braselmann S, Taylor V, Zhao H, Wang S, Sylvain C, Baluom M, et al. R406, an orally available spleen tyrosine kinase inhibitor blocks Fc receptor signaling and reduces immune complex-mediated inflammation. J Pharmacol Exp Ther. 2006;319(3):998-1008.
137. Weinblatt ME, Kavanaugh A, Genovese MC, Musser TK, Grossbard EB, Magilavy DB. An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. N Engl J Med. 2010;363(14):1303-12.
138. Awan FT, Thirman MJ, Patel-Donnelly D, Assouline S, Rao AV, Ye W, et al. Entospletinib monotherapy in patients with relapsed or refractory chronic lymphocytic

leukemia previously treated with B-cell receptor inhibitors: results of a phase 2 study. Leuk Lymphoma. 2019;60(8):1972-7.
139. Jang S, Kwon EJ, Lee JJ. Rheumatoid Arthritis: Pathogenic Roles of Diverse Immune Cells. Int J Mol Sci. 2022;23(2).
140. Toyozaki T, Saito T, Shiraishi H, Tsukamoto Y, Takano H, Nagai T, et al. Macrophage inflammatory protein-1α relates to the recruitment of inflammatory cells in myosin-induced autoimmune myocarditis in rats. Lab Invest. 2001;81(7):929-36.
141. Murayama MA, Shimizu J, Miyabe C, Yudo K, Miyabe Y. Chemokines and chemokine receptors as promising targets in rheumatoid arthritis. Front Immunol. 2023;14:1100869.
142. Ruscitti P, Cipriani P, Carubbi F, Liakouli V, Zazzeroni F, Di Benedetto P, et al. The role of IL-1β in the bone loss during rheumatic diseases. Mediators Inflamm. 2015;2015:782382.
143. Yoshimoto T, Hayashi T, Kondo T, Kittaka M, Reichenberger EJ, Ueki Y. Second-Generation SYK Inhibitor Entospletinib Ameliorates Fully Established Inflammation and Bone Destruction in the Cherubism Mouse Model. J Bone Miner Res. 2018;33(8):1513-9.
144. Poe JC, Jia W, Di Paolo JA, Reyes NJ, Kim JY, Su H, et al. SYK inhibitor entospletinib prevents ocular and skin GVHD in mice. JCI Insight. 2018;3(19).

9. Bibliography of the candidate's publications

The results were based on the following publications:[4]

I. **Káposztás E**, Balogh L, Mócsai A, Kemecsei É, Jakus Z, Németh T. The selective inhibition of the Syk tyrosine kinase ameliorates experimental autoimmune arthritis. Front. Immunol. 2023;14:1279155, Impact factor: 5.7

II. Németh T, Balogh L, **Káposztás E**, Szilveszter KP, Mócsai A. Neutrophil-Specific Syk Expression Is Crucial for Skin Disease in Experimental Epidermolysis Bullosa Acquisita. J Invest. Dermatol. 2023;143(7):1147-56, Impact factor: 5.9

[4]*Káposztás Eszter is the maiden name of Kálmán Eszter.*

10. Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor, Dr. Tamás Németh. His guidance and support have been invaluable in mentoring my research career. I am very thankful for his indispensable advices through my PhD studies and special thank you for him to teaching me to be confident in the life.

I also would like to express my gratitude to Prof. Dr. László Hunyady, the former head of the Physiology Department, and also to Prof. Dr. Attila Mócsai the current head of the institute for their support. Special thanks to Prof. Dr. Péter Enyedi the former chairperson of the Molecular Medicine Program of the PhD School and to Prof. Dr. Péter Várnai, the current chairperson of the program, for their guidance.

I extend my thanks to all members of Translational Rheumatology Research Group. I particularly say thanks to Dr. Lili Balogh, Adrienn Bóka, Nóra Majerhoffer and Zita Panka Nagymihály for their unwavering support and their help in my laboratory work throughout my PhD journey.

Lastly, I am deeply grateful to my whole family for their constant support and encouragement from the very beginning. I could not have reached this milestone without you.