

Accepted Manuscript

Neutrophils: Novel key players in Rheumatoid Arthritis. Current and future therapeutic targets

Irene Cecchi, Ivan Arias de la Rosa, Elisa Menegatti, Dario Roccatello, Eduardo Collantes-Estevez, Chary Lopez-Pedrerera, Nuria Barbarroja



PII: S1568-9972(18)30216-7
DOI: doi:[10.1016/j.autrev.2018.06.006](https://doi.org/10.1016/j.autrev.2018.06.006)
Reference: AUTREV 2221
To appear in: *Autoimmunity Reviews*
Received date: 21 June 2018
Accepted date: 26 June 2018

Please cite this article as: Irene Cecchi, Ivan Arias de la Rosa, Elisa Menegatti, Dario Roccatello, Eduardo Collantes-Estevez, Chary Lopez-Pedrerera, Nuria Barbarroja , Neutrophils: Novel key players in Rheumatoid Arthritis. Current and future therapeutic targets. *Autrev* (2018), doi:[10.1016/j.autrev.2018.06.006](https://doi.org/10.1016/j.autrev.2018.06.006)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Neutrophils: novel key players in Rheumatoid Arthritis. Current and future therapeutic targets

Irene Cecchi^{1,1}, Ivan Arias de la Rosa^{2,1}, Elisa Menegatti¹, Dario Roccatello¹, Eduardo Collantes-Estevez², Chary Lopez-Pedraza², and Nuria Barbarroja^{2,*}
nuria.barbarroja.exts@juntadeandalucia.es

¹Department of Clinical and Biological Sciences, Center of Research of Immunopathology and Rare Diseases - Coordinating Center of Piemonte and Valle d'Aosta Network for Rare Diseases, Turin, Italy

²Rheumatology service, Maimonides Institute for Research in Biomedicine of Cordoba (IMIBIC)/Reina Sofia Hospital/University of Cordoba, Cordoba, Spain

*Corresponding author at: GC05 Group 2^o floor, IMIBIC, Avda. Menendez Pidal s/n, E-14006 Córdoba (SPAIN).

¹ These authors share the first authorship.

Abstract

Rheumatoid Arthritis (RA) is a complex systemic autoimmune disease in which various cell types are involved. Among them, neutrophils have been recognized as important players in the onset and the progression of RA. The pathogenic role of neutrophils in RA lies in the alteration of several processes, including increased cell survival and migratory capacity, abnormal inflammatory activity, elevated oxidative stress and an exacerbated release of neutrophil extracellular traps. Through these mechanisms, neutrophils can activate other immune cells, thus perpetuating inflammation and leading to the destruction of the cartilage and bone of the affected joint.

Given the considerable contribution of neutrophils to the pathophysiology of RA, several studies have attempted to clarify the effects of various therapeutic agents on this subtype of leukocyte.

To date, recent studies have envisaged the role of new molecules on the pathogenic profile of neutrophils in RA, which could represent novel targets in future therapies.

In this review, we aim to review the pathogenic role of neutrophils in RA, the effect of conventional treatments and biologic therapies, and the new, potential targets of neutrophil-derived molecules for the treatment of RA.

Keywords: Rheumatoid Arthritis, treatment, neutrophils, autoimmune disease

1. Introduction

Rheumatoid Arthritis (RA) is a systemic autoimmune disease mainly characterized by chronic joint inflammation, which can promote progressive cartilage and bone damage, thereby leading to increased morbidity and disability.

RA is a multifactorial disorder involving impaired function of both the innate and adaptive immune systems. Moreover, its exact pathogenesis is still unclear.

RA affects the joints directly, since the synovium is the first site of the inflammatory cascade. Synovitis occurs when leukocytes infiltrate the synovial compartment, causing the interaction of synoviocytes with the immune cells [1]. These interactions promote the release of several cytokines, including tumor necrosis factor- α (TNF- α), and interleukin (IL-) -1, IL-6, IL-17 and IL-23 which directly affect the cartilage [2]. In addition, synoviocytes can activate osteoclastogenesis thereby promoting the release of IL-10, of transforming growth factor β (TGF- β), and of IL-6 by osteoclasts, which can act as antigen-presenting cells and activate CD4⁺ and CD8⁺ T cells, thus perpetuating the inflammatory process [3,4].

Among the various cell types involved in the pathogenesis of RA, neutrophils have been acknowledged as important players in the onset and progression of the disease. In fact, neutrophil depletion or functional inhibition have been shown to reduce inflammation and bone damage in experimental models of arthritis [5]. In this review, we describe the pathogenic role of neutrophils in RA, and discuss the effects of various therapies, both conventional and biological agents, on their inflammatory profile and functions. Then we show a number of neutrophil-derived molecules that might serve as novel, potential targets for therapeutic agents.

2. Pathogenic role of neutrophils in RA

The pathogenic role of neutrophils in RA involves an alteration of various processes, including increased migratory capacity and cell survival, abnormal inflammatory activity, enhanced oxidative stress processes, and an exacerbated release of neutrophil extracellular traps (NETs) (Figure 1).

2.1 Migration

Several molecules are involved in the massive recruitment of neutrophils into RA joints, including P-selectin, E-selectin, CXC chemokine receptor 2 (CXCR2), IL-6, CXCL5 and integrin 2b among others [36].

In addition, the effect of the leukotriene (LTB₄) receptor BLT1 and the chemokine receptors CCR1 and CCR2 in favoring neutrophil infiltration is well known. At inflammatory sites, neutrophils can modify their receptor patterns by altering their expression of chemokine receptors and promoting migration into the joint [6]. Moreover, RA neutrophils can induce their own recruitment through the increased synthesis of LTB₄ and IL-1 β [7].

In addition, the Cyr61 pro-inflammatory factor is a protein produced by fibroblast-like synoviocytes, that has been shown to promote neutrophil infiltration through the up-regulation of IL-8 in fibroblasts via the AKT, JNK, ERK1/2 and NF- κ B signaling pathways. Treating collagen-induced arthritis (CIA) mice with anti-Cyr61 has been found to reduce disease activity [8].

2.2 Cell survival

Activated RA neutrophils, especially infiltrated neutrophils, are characterized by a delayed apoptotic process thus increasing the inflammatory status and promoting tissue damage [9]. Neutrophils from synovial fluid have higher levels of the anti-apoptotic protein, Mcl-1 [10]. In addition, a number of studies have reported the role of various molecules and conditions which can regulate neutrophil survival, including leukotriene B₄, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon- γ (IFN- γ), IL-1 β , IL-15, TNF- α , and the local oxygen tensions within the joint [9] [11] [12].

2.3 Inflammation

Upon activation by the autoantibodies and inflammatory mediators that are present in RA, neutrophils release diverse cytokines, chemokines, prostaglandins (PGs) and leukotrienes, leading to an increase in their inflammatory profile. A recent study showed that anti-citrullinated protein antibodies (ACPAs) can act as direct inducers of the inflammatory and pro-oxidative status in circulating neutrophils, which is demonstrated by the increased levels of reactive oxygen species (ROS) and the overexpression of MIP-1 α , MCP-1, IL-8 and IL-23 after *in vitro* treatment of healthy neutrophils with ACPAs isolated from RA patients [13]. Resident neutrophils at synovial sites strongly express IL-17b, therefore enhancing the effects of other cytokines, such as TNF- α , on neutrophil inflammatory function and immune cell migration [14]. IL-22, which seems to promote neutrophil migration through the production of IL-1 α in the affected joints, also plays an important role in the pathogenesis of RA since high levels of this molecule have been found in the synovial tissue of antigen-induced arthritis (AIA) mice at initial

stages [15]. Additionally, IL-20 has recently been described as a pro-inflammatory cytokine with a pathogenic role in RA, and its increased expression in macrophages, neutrophils, synovial fibroblasts and lymphocytes has been correlated with Disease Activity Score 28 (DAS28) and ACPAs positivity [16]. Finally, peripheral and synovial neutrophils from RA patients can produce the B lymphocyte stimulator (BLyS) protein upon TNF- α activation. BLyS is a protein that regulates several features of B-cell physiology, and it has been suggested that it may be a key player in the development of RA since BLyS levels are elevated in RA patients. It has been hypothesized that the release of BLyS from neutrophils can lead to B cell autoimmunity in RA [17].

2.4 The pathogenic intracellular components of neutrophils

A number of the intracellular components of neutrophils have been directly linked to the pathophysiology of RA. Thus, myeloperoxidase (MPO) is the most abundant cytotoxic enzyme found in the azurophilic granules of neutrophils. MPO plays a well-known role in immune responses, since it participates in the activation of T-cells and the elimination of pathogens [18]. Abnormal MPO activity has been reported in several inflammatory conditions, including RA. In fact, depleting MPO in a mouse model with arthritis reduces the severity of the disease [5,19]. In the RA context, high levels of MPO are present in both plasma and synovia, mostly due to the greater generation of NETs [20,21]. MPO triggers the production of inflammatory cytokines and can interact with the endothelial cells, thereby increasing endothelial permeability and activating dendritic cells, both of which are mediated by the consumption of nitric oxide (NO) and the generation of nitrite anion (NO₂⁻) [34,35]. Neutrophil elastase (NE) is a serine-proteinase enzyme which plays an important role in RA as it is specific for many substrates such as elastin, collagen, and fibronectin [24]. NE can activate proteinase-

activated receptors (PARs), leading to the inflammatory response and contributing to the destruction of cartilage [25,26]. A recent study published by Muleyand and colleagues demonstrated that NE can promote damage in the knee joints of mice via PAR2-dependent and activating p44/42 MAPK pathways [27]. Moreover, the enzymatic complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is able to cause cellular stress, and its presence has been linked to inflammatory and autoimmune conditions [28]. RA synovial neutrophils can show increased NADPH oxidase activity by phosphorylation of p47phox (NCF1 or neutrophil cytosolic factor 1). This enzyme may release superoxide anion (O_2^-) thus contributing to excessive ROS concentrations, amplifying inflammatory reactions and promoting tissue damage [29].

2.5 Neutrophil extra-cellular traps

NETs are extracellular fibrous networks composed of nuclear and granular proteins which protrude from the membrane of activated neutrophils [30]. NETs are found in a wide range of pathologic conditions, and they have recently been implicated in the pathogenesis of many autoimmune and rheumatic diseases, including RA [31,32]. Several studies have demonstrated a high degree of spontaneous NETs formation in circulating and synovial RA neutrophils, showing a strong correlation between free circulating DNA levels and inflammatory markers such as C-reactive protein (CRP), erythrocyte sedimentation rate, ACPAs titers, and levels of IL-17 [33–35]. In addition, an impairment in the NETs degradation machinery is found in RA patients mainly due to a decrease in the DNase activity [35,36].

It has been demonstrated that the formation of NETs can accelerate disease progression [33,37]. Specifically, the release of NETs can contribute to the generation of ACPAs, leading to the production of inflammatory molecules such as IL-6, IL-8, chemokines

and adhesion proteins [33,38]. ACPAs can also promote the release of peptidylarginine deiminases (PADIs) from neutrophils, which in turn can catalyze the modification of arginine to citrulline, creating a vicious circle of autoantibody production [39]. Moreover, NETs contain an important deposit of PADIs that can be accumulated in the synovial fluid thus promoting the formation of citrullinated products in RA [40]. Furthermore, RA patients have elevated levels of low-density granulocytes (LDGs) in the peripheral blood, a newly discovered subset of cells with a phenotype of CD14^{dim} and CD15^{bright} that are more prone to suffer NETosis [41,42]. Given this evidence, NETs have been proposed as a potential novel biomarker since the plasma levels of cell-free nucleosomes show high specificity (92%) and sensitivity (91%) for the diagnosis of RA [35] and, even more interestingly, for the diagnosis of “early arthritis” [34].

3. Effect of therapies on neutrophils in the RA setting

The outcome and management of RA have greatly improved over the last 30 years due to the availability of a wide range of new therapeutic tools, combined with the systematic use of non-pharmacological approaches, earlier diagnosis, and close follow-up. Nowadays, the treatment of RA aims to achieve long-lasting remission or at least low levels of disease activity.

The pharmacological management of RA includes the cautious use of glucocorticoids (GCs), the use of non-steroidal anti-inflammatory drugs (NSAIDs), and of disease-modifying anti-rheumatic drugs (DMARDs), including conventional synthetic DMARDs (csDMARDs), biological (bDMARDs), and targeted synthetic DMARDs (tsDMARDs) [43–45]. Considering the relevant contribution of neutrophils in the pathophysiology of RA, several studies have tried to clarify at least the indirect effects of the various therapeutic agents on this subtype of leukocyte (Table 1).

3.1 NSAIDs

NSAIDs are effective -choose at relieving pain and improving stiffness in RA patients, but they are not able to prevent structural damage of the cartilage and bone. The main mechanism of action of all NSAIDs is the inhibition of prostaglandins (PGs) production from arachidonic acid (AA) through the blockade of the two main isoforms of cyclooxygenase (COX), COX-1 and COX-2 [46]. Interestingly, recent studies have brought to light the existence of various non-PGs-mediated anti-inflammatory effects [47-49].

NSAIDs have the ability to inhibit the adhesion process of neutrophils, which represents a crucial step in the inflammatory cascade. In detail, a number of NSAIDs are able to interfere with the function of adhesion molecules such as L-selectin, both *in vitro* and *in vivo*, or through the modulation of the function of integrin CD11b/CD18 [50][51][52][53] [9], resulting in the reduction of the adhesive properties of these cells, and consequently increasing the anti-inflammatory effects of this class of therapeutic agents. In addition, NSAIDs prevent neutrophil chemotaxis and migration [55], inhibit degranulation [56], decrease the levels of elastase and production [57], and promote neutrophil apoptosis [58].

A recent study by Lapponi and colleagues further demonstrated that treating neutrophils with acetylsalicylic acid inhibits NETs formation through the phosphorylation of NF- κ B [59].

However, several aspects of the effects of NSAIDs on neutrophils remain unclear, especially in the RA setting.

3.2 Glucocorticoids

Despite the serious concerns regarding the long-term safety of GCs, their use proved to be effective at low doses in the early stages of RA, as a short-term therapy when added to csDMARDs, and as an essential therapeutic option in case of disease flares.

Several studies have provided insight into the effects of GCs on immune cells, and specifically on neutrophils. In general, GCs increase the number of circulating neutrophils mainly through the mobilization of the marginated pool [61], inhibit chemotaxis [62][63][64], decrease the adherence capacity [65][66][67] even if some studies have shown contradictory results [68], delay apoptosis in circulating neutrophils [69][70], and reduce the production of ROS [71][72], degranulation, and the release of inflammatory mediators.

Very little is known about the relationship between the use of GCs and NETs formation, but a recent study showed no effect of treatment with dexamethasone on cultured neutrophils isolated from healthy donors (HDs) stimulated either with phorbol 12-myristate 13-acetate (PMA) or with TNF- α [59].

In the specific RA setting, low doses of prednisone do not seem to have a significant impact on the circulating neutrophil count, however methylprednisolone decreases their adhesion capacity and impairs trafficking into inflamed joints [73][74][75].

Despite the widespread use of GCs in the treatment of RA, their mechanism of action on neutrophils is not fully understood, mainly due to the high complexity of the physiopathologic role exerted by this subtype of leukocyte in the disease.

3.3 Leflunomide

Leflunomide (LFN) is one of the main therapeutic agents recommended by both the American College Of Rheumatology in 2008 and by the European League Against Rheumatism (EULAR) for the treatment of RA [76][77].

LFN is a synthetic isoxazole-derivative oral pro-drug with multiple therapeutic possibilities [78][79][80][81]. Its active metabolite A-771726 (teriflunomide) inhibits the pyrimidine synthesis and several cellular protein kinases, resulting in an immunomodulatory effect. However, the exact underlying mechanism of action is largely unknown.

LFN mainly acts by decreasing the activity and proliferation of lymphocytes both in *in vitro* and in *in vivo* studies [82][83], and lowering the concentration of inflammatory mediators blocking the production of ROS from leukocytes [84]. To date, the specific effect of LFN on neutrophils has been poorly investigated. To our knowledge, only one previous study reported that LFN is able to reduce neutrophil adhesion and oxidative burst, in addition to a rapid effect on neutrophil chemotaxis, resulting in decreased migration of these cells into the affected joints [85].

3.4 Methotrexate

Methotrexate (MTX) is considered a cornerstone and the main starting therapy for RA. Growing evidence suggests that one of the main mechanisms by which MTX exerts its anti-inflammatory effect is by increasing the level of adenosine through the up-regulation of its receptors on neutrophils. This leads to a decrease in the production of cytokines, especially TNF- α and IL-1 β , the suppression of NF-kB activation, and to a diminished accumulation of leukocytes at inflamed sites in *in vivo* experiments [86][87][88].

As described for LFN, previous *in vitro*, *ex vivo* and *in vivo* studies demonstrated that MTX significantly inhibits neutrophil chemotaxis in RA patients through a reversible mechanism [89][90], which leads to a decrease in cell trafficking into the inflamed joints.

MTX has proven to be effective in inducing apoptosis of circulating neutrophils from polyarthritis patients with less than 6 weeks of disease duration [91], as both circulating and synovial neutrophils show a delay in spontaneous apoptosis in RA patients when compared with HDs [92][93]. Using an adjuvant induced arthritis (AIA) animal model, Novaes and co-workers proved that a low dose of MTX (0.25 mg/kg) decreases the intra-articular levels of PG-E2 and thromboxane B2 as compared to the saline-treated controls [94].

Moreover, Richard and colleagues demonstrated that MTX is effective in decreasing, *ex vivo*, the synthesis of LTB4 by neutrophils from patients with active RA before 6-8-weeks of disease duration with a parallel inhibition of the 5-lipoxygenase (5-LO) [95].

3.5 Anti-TNF- α

The prominent role of TNF- α in the pathogenesis and progression of RA is well established. This evidence has led to the development of a new class of bDMARDs which represent a breakthrough in the treatment of this disease. Despite their heterogeneity, the main mechanism of action of these drugs relies on the ability to block the biologic activity of TNF- α . As previously described, this cytokine has pleiotropic effects on inflammation and neutrophil functions, such as priming the neutrophil respiratory burst, increasing the expression levels of other cytokines, chemokines and adhesion molecules, and stimulating ROS production [96][97][98][99].

Indeed, TNF- α -blockade agents proved to be effective in deactivating the proinflammatory cytokine cascade with chemotactic function, such as IL-8 and IL-33, decreasing the expression of adhesion molecules, and lowering cell-trafficking into inflamed joints in the specific setting of RA [100][101]. Clinical responders to TNF- α -inhibitors display a decrease in the expression of adhesion molecules and chemotaxis,

along with a reduction in the concentration of chemokines [102]. In line with these results, the beneficial effect of anti-TNF- α agents on the neutrophil inflammatory profile was further confirmed in 2005 when Wittkowski and co-workers demonstrated that RA patients who were clinical responders to infliximab showed a decrease in neutrophil activation during synovial inflammation. This was reflected by the lower serum concentration and synovial expression of the pro-inflammatory calcium-binding protein S100A12, which is part of the group of damage-associated molecular pattern molecules [103].

A subsequent study proved that a fully human anti-TNF- α monoclonal antibody can actually decrease leukocyte influx from the peripheral blood into inflamed joints, but *ex vivo* chemotaxis and superoxide production did not decrease significantly after anti-TNF- α administration [104].

Moreover, a few studies have provided novel insight into the modulation of NETs formation in RA patients in response to treatment. In detail, a recent paper by Pérez-Sánchez et al. demonstrated that along with an improvement in disease activity and a decrease of inflammatory mediators, after 6 months of therapy with infliximab RA patients showed a parallel reduction in the generation of NETs [35].

In conclusion, several studies have shown the numerous effects of anti-TNF- α agents on neutrophil functions, even if they do not represent the specific target of this group of monoclonal antibodies. Indeed, the precise effect of TNF- α -inhibitors has not yet been fully elucidated and further studies are needed in order to assess the mechanisms through which these therapeutic agents can modulate neutrophil functions and change their inflammatory profile.

3.6 Anti-IL6 receptor

Tocilizumab (TCZ) is a humanized monoclonal antibody of the IgG1 subclass that targets the soluble and membrane-bound IL-6 receptor (IL-6R), therefore inhibiting the proinflammatory cascade driven by IL-6. It proved to be effective in controlling disease activity and radiography-proven progression of RA patients, as monotherapy and in combination with other csDMARDs [67].

IL-6 is a pro-inflammatory cytokine that is implicated in a wide range of chronic conditions, and it shows an extensive range of effects in the RA context associated both with systemic and local manifestations of the disease. Together with T-cells and macrophages, neutrophils (especially when they are activated) release IL-6R, resulting in trans-signaling in other immune cells that do not express this receptor [68]. Despite this evidence, the specific effect of TCZ on neutrophil functions has been poorly investigated.

A number of clinical trials showed that TCZ *in vivo* can cause acute but transient dose-dependent neutropenia [69], which might represent a marker of response to treatment [70], but it does not seem to be associated with the occurrence of severe infections [71]. The mechanism through which TCZ is able to induce a decrease in neutrophil count is unclear, and several studies have provided contradictory results regarding the ability of the IL-6 blockade to induce apoptosis [72][73][74][75].

TCZ has also been found to reduce ROS generation and phagocytosis under hypoxic conditions as can be observed in inflamed joints. Furthermore, the IL-6 blockade modulates the neutrophil production of cytokines and chemokines in a divergent way based on the presence or absence of hypoxia, which induces neutrophil recruitment in inflamed joints without enhancing their survival [76].

TCZ also proved to be effective in inhibiting NETs formation in parallel with clinical and serological improvements in RA patients [66] [77].

3.7 Rituximab

Rituximab (RTX) was licensed for the treatment of RA patients who are refractory/intolerant to at least one TNF- α -inhibitor, and thereafter it was found to be effective in bDMARDs-naïve patients and in the early stages of the disease [71] [72]. RTX is a chimeric anti-CD20 monoclonal antibody which, *in vivo*, mainly induces an acute but transient B-cell depletion in the blood and partially in the bone marrow and synovial tissue [73] [74] [75]. A retrospective analysis performed by Tesfa and colleagues demonstrated that the incidence of neutropenia in autoimmune diseases seems to be less frequent than in onco-hematologic disorders, since it is 3% for RA [77]. One of the possible mechanisms through which RTX causes this dangerous adverse effect is the indirect arrest of the maturation at the promyelocytic stage of granulopoiesis [78]. Other possible mechanisms have been suggested, including an immune-mediated effect, a delayed efflux of neutrophils from the bone marrow due to the change in homeostasis.

3.8 Tofacitinib

Tofacitinib is an oral Janus-Kinase Inhibitor (JAK3/JAK1) that has been approved for use both as monotherapy or in combination with csDMARDs for the treatment of RA patients with moderate to severe disease activity who are refractory to DMARDs [105]. The mechanism of action of Tofacitinib is different compared to other DMARDs as it works at the intracellular level. Tofacitinib is a reversible, competitive inhibitor of phosphorylation and therefore of the activation of JAK, and it is known to exert its effect directly on T-cell proliferation and functions. On the contrary, the specific effect of Tofacitinib on neutrophils has been poorly investigated. A limited number of clinical trials have reported that Tofacitinib can moderately affect the absolute neutrophil count [114, 116, 117] in a dose-dependent way, usually remaining in the range of normality,

reaching a plateau within 3 months of therapy and then recovering after drug discontinuation [109]. This phenomenon may represent the consequence of the overall modulation of inflammation, and therefore can be useful for monitoring the therapeutic response to the treatment. More recently, a study by Mitchell and colleagues tried to better define the direct effect of Tofacitinib on neutrophil functions. As a matter of fact, a number of studies have reported an increased rate of infections in RA patients treated with this agent compared to patients treated with anti-TNF- α or placebo [110][111][108][112]. Tofacitinib inhibited the anti-apoptotic effect of GM-CSF and IFN- γ on neutrophils from HDs. In addition, after incubating Tofacitinib for 20 hours at the highest concentration (200 ng/ml) with neutrophils from RA patients stimulated with GM-CSF, this therapeutic agent significantly inhibited apoptosis. Tofacitinib was also found to significantly prevent *ex-vivo* random migration of the neutrophils isolated from RA patients. No inhibitory effect of Tofacitinib on ROS production by neutrophils of RA patients was reported [113]. In summary, recent findings showed the effects of the JAK-inhibitor, Tofacitinib on neutrophil functions and number, which is the result of a complex system of interactions between immune-cells, chemokines and cytokines.

4. Neutrophil-derived molecules as emerging therapies for RA

Despite the great progress that has been made in the last decades on the treatment and management of RA, especially with the introduction of biological agents, only two thirds of RA patients achieve a satisfactory response. The development of new drugs whose mechanisms of action not rely only on TNF- α inhibition and autoantibody production and which could fill the lack of effectiveness of the standard therapeutic regimens, represents an urgent, unmet clinical need and it must be addressed. Therefore, neutrophils and their functions represent appealing potential targets for future therapeutic strategies (Table 2).

4.1 Leukotriene B4

Leukotrienes are a family of lipid mediators that play a key role in the pathogenesis of inflammation. They are synthesized in the leukocytes from arachidonic acid (AA) via the action of 5-lipoxygenase (5-LO). Among them, leukotriene B4 (LTB₄) is recognized as the most potent chemoattractant of leukocytes. High levels of LTB₄ are present in the serum of RA patients thus leading to neutrophil activation and recruitment at inflammation sites, correlating with the severity of the disease [114][115][116].

The importance of LTB₄ in arthritis has been demonstrated by the fact that genetic ablation of its receptor BLT1 is able to prevent the arthritis in collagen induced arthritis (CIA) mice [117][118]. In addition, antagonists of the LTB₄ receptor can reduce clinical symptoms and histological changes in murine CIA [119][120].

Several LO inhibitors are being tested as potential candidates for effective therapies in RA. To date, only one drug, named zileuton, has been tested for clinical use [121]. However, its efficacy and medical acceptance have been compromised by a suboptimal pharmacokinetic and pharmacodynamic profile [122] and possible hepatotoxicity [123]. Thus, there is a need for a more potent, better tolerated, non-hepatotoxic 5-LO inhibitor that could maximize the benefits of inhibiting the leukotriene pathway and provide greater efficacy than what was obtained with zileuton and leukotriene receptor antagonists. In this sense, PF-41911834 is a novel 5-LO inhibitor [124] which was found to reduce arthritis-associated pain and inflammation in a rat model [124] through the TNF- α pathway [125].

Other inhibitors of LO such as esculetin are being studied in preclinical models, and have shown that esculetin is a powerful chemotactic agent influencing neutrophil migration and reducing neutrophil infiltration in animal models of inflammation [126].

Esculetin may effectively modulate the LTB₄ levels in adjuvant-induced arthritis in rats and thus may be considered an interesting drug candidate for patients with RA [127].

4.2 CC chemokine receptor type 2

CC chemokine receptor type 2 (CCR2) is a chemokine receptor expressed on monocytes, T and B cells and immature dendritic cells. Under physiological conditions it is not present on the surface of neutrophils, but its expression is induced in inflammatory conditions.

Several data from human and animal models support the role of CCR2 in the development of RA. Thus, CCR2 genetic polymorphisms modulate the risk of developing arthritis in patients with psoriasis [128]. In mice, the administration of anti-CCL2 monoclonal antibodies before disease onset in an MRL/lpr model was found to prevent the onset of arthritis [129]. Thereafter, the blockade of CCR2 ameliorated disease activity during the onset of CIA thus supporting the therapeutic benefits in the early stages of RA [130].

RA neutrophils, both peripheral and infiltrated, highly express CCR2) [131][6]. In addition, modulation of the expression of CCR2 by pharmacological blockade inhibited the migration of these cells into the inflamed tissue in mice, demonstrating a novel chemokine responsiveness at inflammation sites [6]. Likewise, high concentrations of CCL2, a CCR2 ligand, are found in the synovial fluid of RA patients [132], and neutrophils from RA patients express high amounts of this molecule [13]. In RA patients, CCR2 inhibitors (anti-CCL2 monoclonal antibody) failed to show any beneficial effects in patients with established disease during phase II trials [133][134]. This lack of efficacy might be explained by the impairment of immunosuppressive mechanisms related to the role of CCR2 on regulatory T-cells. All the studies that have been performed so far show the importance of CCR2 for recruiting neutrophils into the

joints during the acute phase of RA. These findings could reinforce the possibility that CCR2 inhibitors might be tested as a potential adjuvant therapy in RA patients at early stages and in case of disease flare.

4.3 CXCR1/CXCR2

CXCR1/CXCR2 are chemokine receptors that mediate neutrophil accumulation and activation at the site of inflammation and infection. CXCR1 is specific for CXCL8, whereas CXCR2 also interacts with CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL7 [135]. It has been shown that CXCL1 and CXCL5 mediate LTB₄-dependent recruitment of neutrophils to inflamed joints in AIA mice [136]. Moreover, levels of CXCL1 and CXCL5 are elevated in the synovial fluid of RA patients indicating its role in inflammatory joint disease.

The relevance of the function of these chemokine receptors in RA was first shown when treatment with DF2162, a novel, orally-active, non-competitive allosteric inhibitor of CXCR1 and CXCR2, significantly ameliorated AIA in rats. The effects of this inhibitor were considered by the authors to be quantitatively and qualitatively similar to those observed after anti-TNF α antibody treatment [137]. In addition, this treatment greatly decreased the influx of neutrophils into the knee joint and the periarticular tissue after antigen challenge in immunized mice [138].

Targeting neutrophil migration with the CXCR2/CXCR1 antagonist, SCH563705, led to a dose-dependent decrease in clinical disease assessment scores, paw thickness measurements and clearly reduced inflammation, and bone and cartilage degradation [139]. To date, no clinical trials have been developed.

4.4 Interleukins

4.4.1 IFNLR1-IL28RA

IFN- ω 1, - ω 2, and - ω 3 (or IL-29, IL-28A, and IL-28B, respectively) are members of the class II cytokine family evolutionarily related to both IL-10 and type I IFNs (IFN- α/β), and collectively referred to as type III IFNs. Recently, it has been shown that neutrophils can express elevated levels of IFN-receptor 1 (IFNLR1)-IL-28 receptor (IL-28RA), a specific receptor of IL-28, also known as IFN- ω 2 [140].

Despite the use of a different receptor complex, IFNLR1-IL28RA activates similar signaling pathways to those of the type I IFN receptor [141] [142]. IFN- ω s have immunomodulatory effects, such as proliferation of regulatory T-cells [143] and down-regulation of Th2 cell-mediated inflammation [144].

Of note, synovial neutrophils from the joints of CIA mice display high levels of IL-28RA mRNA[140]. Treatment with IL-28A reduced the levels of LTB4 receptor in neutrophils, restricting their recruitment into the joint and inhibiting the local neutrophil migratory capacity in mice. In addition, it reduced the levels of IL-1 produced produced by neutrophils, but not by other cell types, suggesting the specificity of IL-28A to target neutrophils.

IL-1 secretion by neutrophils is believed to be critical for the amplification of the arthritic process through the induction of neutrophil-active chemokines by synovial cells [118]. These data indicate that agonists of IFNLR1-IL-28 can have potential as novel therapeutics, since treatment with IL-28A specifically targets IL-1 β -expressing neutrophils, inhibiting both their migratory capacity to the joint and amplification of the inflammation.

4.4.2 Interleukin-33

IL-33 (or IL1F11) is the most recently described cytokine of the IL-1 family, which includes 11 members classified as IL-1F1 to IL-1F11[145][146]. It is a chromatin-associated nuclear cytokine that is mainly produced by fibroblast-like synoviocytes and

macrophages. IL-33 transduces its signal through the heterodimeric receptor, the α -chain IL-33R2 and the β -chain IL-1RAcP4, promoting the activation of a number of intracellular kinases such as MAP kinases p38, ERK1/2, JNK1/2 and the transcription factor NF- κ B [146][147].

The relevant role of the IL-33-IL-33R pathway in RA has been highlighted by the fact that soluble IL-33R was found to be a potent inhibitor of arthritis development in CIA mice [148]. IL-33 contributes to the development of local inflammation in a murine model of RA via IFN γ [149].

The specific role of IL-33 on neutrophils has recently been observed using various models of arthritis. Thus, IL-33R knockdown mice showed reduced neutrophil migration to the knee joint. In addition, local and systemic treatment of AIA mice with sIL-33R (the decoy receptor of IL-33) inhibited neutrophil migration. *In vitro* experiments performed on purified neutrophils demonstrated the direct effect of IL-33 in inducing neutrophil chemotaxis [150]. The authors suggested that IL-33 can promote the influx of neutrophils to the site of antigen challenge by two mechanisms: an indirect one, through the activation of synoviocytes and macrophages to produce TNF- α , IL-1 β , CXCL1 and CCL3 which induce the recruitment of neutrophils to the joint, and the other by directly attracting neutrophils to the site of inflammation [150].

Thereafter, it was demonstrated that IL-33 activity could be increased by neutrophil proteases that are released during the inflammation process. Thus, through *in vivo* and *in vitro* studies, it was shown that serine proteases cathepsin G and elastase could cleave full-length IL-33, generating mature forms with higher activity in human activated neutrophils [151].

Thus, targeting IL-33 may represent a novel strategy against RA by inhibiting neutrophil accumulation.

4.4.3 Peptidylarginine deiminase 4

PADI genes encode PAD proteins that convert arginine residues to citrulline. Specifically, PADI type 4 (PADI4) plays a major role in epigenetic regulation via the citrullination of histones and transcription factors. It is mainly expressed in myeloid lineage cells, including neutrophils, and its expression is inducible under inflammatory conditions. The multifaceted role of PADI4 in the immune function inducing cytokine production, activating pro-inflammatory Th1 and Th17 responses and inhibiting Th2 functions and promoting the maturation of dendritic cells is well-known [152][153][154][155]. Moreover, it was shown that knockout PADI4 mice had a reduced number of neutrophils and monocytes, while the number of B- or T-cells remained unchanged, suggesting that PADI4 controls the survival of myeloid lineage cells [156].

The increased and uncontrolled production of citrullinated antigens results in the development of ACPAs in RA. Thus, PADI4 has been identified as an RA susceptibility gene in a large-scale, case-control association study using a gene based genome-wide association study method [157]. The relevance of the PADI4 gene on arthritis was demonstrated by a recent study which showed that PADI4 knockdown CIA mice had a lower clinical disease activity score [158].

With regard to neutrophils, PADI4 may also influence the immune response and inflammation by triggering the release of NETs. Thus, PADI activity is critical for NETs formation [159][160]. As NETs formation is a process that is exacerbated in RA, PADI4 is released extracellularly by the activated neutrophils in the joints thereby increasing cell activation and cytokine production, and eventually perpetuating inflammation [161].

In recent preclinical studies, the treatment of CIA mice using a selective, small molecule inhibitor of PADI4 (GSK199) prevented the worsening of clinical and histological disease severity [162]. PADI4 inhibitors further represent an anti-NET target that may be an effective, novel therapy for the treatment of RA. To date, clinical trials have not been conducted in RA patients.

Take-home messages

- 1- Neutrophils are not only involved in the innate immune response, but they are also key players in the pathogenesis and progression of RA, perpetuating systemic inflammation thus leading to joint destruction and patient disability.
- 2- To date, there is no treatment that specifically targets neutrophils, but the current standard therapeutic approaches show an indirect beneficial effect on their pathogenic profile. Although limited, previous studies showed that NSAIDs and csDMARDs are able to prevent neutrophil chemotaxis and migration, decrease degranulation and ROS production, while the main ability of bDMARDs is to lower the production of inflammatory mediators and prevent the release of NETs. Further studies focused on the specific effect of these therapies, especially bDMARDs, on RA neutrophils are necessary.
- 3- There are a number of novel molecules involved in several neutrophil functions, including cell-activation and trafficking, such as LTB₄, chemokine receptors (CCR2, CXCR1/CXCR2), interleukins (IFNLR1-IL28RA, IL-33) and PADI4, which represent relevant potential targets for future treatment of RA.

Conflict of interest

All authors state that they have no current or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

Funding

This work was supported by grants from the 'Junta de Andalucía' (CTS-7940, PI-0191-2013), the Ministry of Health (ISCIII) co-financed with FEDER funds (PI15/01333, CP15/00158, PI17/01316 and RD16/0012/0015). CL-P was supported by a contract from the Spanish Junta de Andalucía. NB was supported by a Ministry of Health postdoctoral fellowship (Miguel Servet Program).

Acknowledgements

The authors acknowledge the excellent assistance of A. Reifs with the art work.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

- [1] Picerno V, Ferro F, Adinolfi A, Valentini E, Tani C, Alunno A. One year in review: The pathogenesis of rheumatoid arthritis. *Clin Exp Rheumatol* 2015;33:551–8.
- [2] Karmakar S, Kay J, Gravalles EM. Bone Damage in rheumatoid arthritis: Mechanistic insights and approaches to prevention. *Rheum Dis Clin North Am* 2010;36:385–404. doi:10.1016/j.rdc.2010.03.003.
- [3] Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF- α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest* 2000;106:1481–8. doi:10.1172/JCI11176.
- [4] Li H, Hong S, Qian J, Zheng Y, Yang J, Yi Q. Crosstalk between the bone and immune systems: osteoclasts function as antigen-presenting cells and activate CD4⁺ and CD8⁺ T cells. *Blood* 2010;116:210–7. doi:DOI 10.1182/blood-2009-11-255026.
- [5] Tanaka D, Kagari T, Doi H, Shimosato T. Essential role of neutrophils in anti-type II collagen antibody and lipopolysaccharide-induced arthritis. *Immunology* 2006;119:195–202. doi:10.1111/j.1365-2567.2006.02424.x.
- [6] Talbot J, Bianchini FJ, Nascimento DC, Oliveira RDR, Souto FO, Pinto LG, et al. CCR2 expression in neutrophils plays a critical role in their migration into the joints in rheumatoid arthritis. *Arthritis Rheumatol* 2015;67:1751–9. doi:10.1002/art.39117.
- [7] Sadik CD, Kim ND, Iwakura Y, Luster AD. Neutrophils orchestrate their own

- recruitment in murine arthritis through C5aR and Fc R signaling. *Proc Natl Acad Sci* 2012;109:E3177–85. doi:10.1073/pnas.1213797109.
- [8] Zhu X, Xiao L, Huo R, Zhang J, Lin J, Xie J, et al. Cyr61 is involved in neutrophil infiltration in joints by inducing IL-8 production by fibroblast-like synoviocytes in rheumatoid arthritis. *Arthritis Res Ther* 2013;15:R187. doi:10.1186/ar4377.
- [9] Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology* 2010;49:1618–31. doi:10.1093/rheumatology/keq045.
- [10] Dzhagalov I, St John A, He Y-W. The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood* 2007;109:1620–6. doi:10.1182/blood-2006-03-013771.
- [11] Barcellos-de-Souza P, Canetti C, Barja-Fidalgo C, Arruda MA. Leukotriene B(4) inhibits neutrophil apoptosis via NADPH oxidase activity: redox control of NF- κ B pathway and mitochondrial stability. *Biochim Biophys Acta* 2012;1823:1990–7. doi:10.1016/j.bbamcr.2012.07.012.
- [12] Cross A, Barnes T, Bucknall RC, Edwards SW, Moots RJ. Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints. *J Leukoc Biol* 2006;80:521–8. doi:10.1189/jlb.0306178.
- [13] Barbarroja N, Pérez-Sánchez C, Ruiz-Limon P, Castro-Villegas C, Aguirre MA, Carretero R, et al. Anticyclic citrullinated protein antibodies are implicated in the development of cardiovascular disease in rheumatoid arthritis. *Arterioscler Thromb Vasc Biol* 2014;34:2706–16. doi:10.1161/ATVBAHA.114.304475.
- [14] Kouri VP, Olkkonen J, Ainola M, Li TF, Björkman L, Kontinen YT, et al.

- Neutrophils produce interleukin-17B in rheumatoid synovial tissue. *Rheumatol (United Kingdom)* 2014;53:39–47. doi:10.1093/rheumatology/ket309.
- [15] Pinto LG, Talbot J, Peres RS, Franca RF, Ferreira SH, Ryffel B, et al. Joint production of IL-22 participates in the initial phase of antigen-induced arthritis through IL-1 β production. *Arthritis Res Ther* 2015;17:235. doi:10.1186/s13075-015-0759-2.
- [16] Šenolt L, Prajzlerová K, Hulejová H, Šumová B, Filková M, Veigl D, et al. Interleukin-20 is triggered by TLR ligands and associates with disease activity in patients with rheumatoid arthritis. *Cytokine* 2017;97:187–92. doi:10.1016/j.cyto.2017.06.009.
- [17] Assi LK, See HW, Ludwig A, Raza K, Gordon C, Salmon M, et al. Tumor necrosis factor ?? activates release of B lymphocyte stimulator by neutrophils infiltrating the rheumatoid joint. *Arthritis Rheum* 2007;56:1776–86. doi:10.1002/art.22697.
- [18] Strzepa A, Pritchard KA, Dittel BN. Myeloperoxidase: A new player in autoimmunity. *Cell Immunol* 2017;317:1–8. doi:10.1016/j.cellimm.2017.05.002.
- [19] Odobasic D, Kitching AR, Yang Y, O’Sullivan KM, Muljadi RCM, Edgton KL, et al. Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function. *Blood* 2013;121:4195–204. doi:10.1182/blood-2012-09-456483.
- [20] Odobasic D, Yang Y, Muljadi RCM, O’Sullivan KM, Kao W, Smith M, et al. Endogenous Myeloperoxidase Is a Mediator of Joint Inflammation and Damage in Experimental Arthritis. *Arthritis Rheumatol* 2014;66:907–17. doi:10.1002/art.38299.

- [21] Fernandes RMSN, Silva NP da, Sato EI. Increased myeloperoxidase plasma levels in rheumatoid arthritis. *Rheumatol Int* 2012;32:1605–9. doi:10.1007/s00296-011-1810-5.
- [22] Eiserich JP, Baldus S, Brennan M-L, Ma W, Zhang C, Tousson A, et al. Myeloperoxidase, a Leukocyte-Derived Vascular NO Oxidase. *Science* (80-) 2002;296:2391–4. doi:10.1126/science.1106830.
- [23] Klinke A, Nussbaum C, Kubala L, Friedrichs K, Rudolph TK, Rudolph V, et al. Myeloperoxidase attracts neutrophils by physical forces. *Blood* 2011;117:1350–8. doi:10.1182/blood-2010-05-284513.
- [24] Watanabe H, Hattori S, Katsuda S, Nakanishi I, Nagai Y. Human neutrophil elastase: degradation of basement membrane components and immunolocalization in the tissue. *J Biochem* 1990;108:753–9.
- [25] Busso N, Frasnelli M, Feifel R, Cenni B, Steinhoff M, Hamilton J, et al. Evaluation of protease-activated receptor 2 in murine models of arthritis. *Arthritis Rheum* 2007;56:101–7. doi:10.1002/art.22312.
- [26] Hollenberg MD, Mihara K, Polley D, Suen JY, Han A, Fairlie DP, et al. Biased signalling and proteinase-activated receptors (PARs): targeting inflammatory disease. *Br J Pharmacol* 2014;171:1180–94. doi:10.1111/bph.12544.
- [27] Muley MM, Reid AR, Botz B, Bölcskei K, Helyes Z, McDougall JJ. Neutrophil elastase induces inflammation and pain in mouse knee joints via activation of proteinase-activated receptor-2. *Br J Pharmacol* 2016;173:766–77. doi:10.1111/bph.13237.
- [28] Panday A, Sahoo MK, Osorio D, Batra S. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cell Mol Immunol*

- 2015;12:5–23. doi:10.1038/cmi.2014.89.
- [29] El-Benna J, Dang PM-C, Gougerot-Pocidallo MA, Marie JC, Braut-Boucher F. p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. *Exp Mol Med* 2009;41:217–25. doi:10.3858/emm.2009.41.4.058.
- [30] Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007;176:231–41. doi:10.1083/jcb.200606027.
- [31] Lee KH, Kronbichler A, Park DD-Y, Park Y, Moon H, Kim H, et al. Neutrophil extracellular traps (NETs) in autoimmune diseases: A comprehensive review. Elsevier B.V; 2017. doi:10.1016/j.autrev.2017.09.012.
- [32] Corsiero E, Pratesi F, Prediletto E, Bombardieri M, Migliorini P. NETosis as source of autoantigens in rheumatoid arthritis. *Front Immunol* 2016;7:1–9. doi:10.3389/fimmu.2016.00485.
- [33] Khandpur R, Carmona-rivera C, Vivekanandan-giri A, Yalavarthi S, Knight JS, Friday S, et al. RA_ACPA_NETs_SciTranMed2013. *Sci Transl Med* 2013;5. doi:10.1126/scitranslmed.3005580.NETs.
- [34] Sur Chowdhury C, Giaglis S, Walker UA, Buser A, Hahn S, Hasler P. Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther* 2014;16:R122. doi:10.1186/ar4579.
- [35] Pérez-Sánchez C, Ruiz-Limón P, Aguirre MA, Jiménez-Gómez Y, Arias-de la Rosa I, Ábalos-Aguilera MC, et al. Diagnostic potential of NETosis-derived products for disease activity, atherosclerosis and therapeutic effectiveness in

- Rheumatoid Arthritis patients. *J Autoimmun* 2017;82:31–40. doi:10.1016/j.jaut.2017.04.007.
- [36] Bonanni A, Vaglio A, Bruschi M, Sinico RA, Cavagna L, Moroni G, et al. Multi-antibody composition in lupus nephritis: Isotype and antigen specificity make the difference. *Autoimmun Rev* 2015;14:692–702. doi:10.1016/j.autrev.2015.04.004.
- [37] Kahlenberg JM, Carmona-Rivera C, Smith CK, Kaplan MJ. Neutrophil Extracellular Trap-Associated Protein Activation of the NLRP3 Inflammasome Is Enhanced in Lupus Macrophages. *J Immunol* 2013;190:1217–26. doi:10.4049/jimmunol.1202388.
- [38] Thieblemont N, Wright HL, Edwards SW, Witko-Sarsat V. Human neutrophils in auto-immunity. *Semin Immunol* 2016;28:159–73. doi:10.1016/j.smim.2016.03.004.
- [39] Jones JE, Causey CP, Knuckley B, Slack-Noyes JL, Thompson PR. Protein arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. *Curr Opin Drug Discov Devel* 2009;12:616–27.
- [40] Spengler J, Lugonja B, Jimmy Ytterberg A, Zubarev RA, Creese AJ, Pearson MJ, et al. Release of active peptidyl arginine deiminases by neutrophils can explain production of extracellular citrullinated autoantigens in rheumatoid arthritis synovial fluid. *Arthritis Rheumatol* 2015;67:3135–45. doi:10.1002/art.39313.
- [41] Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis. *Nat Rev Rheumatol* 2014;10:593–601. doi:10.1038/nrrheum.2014.80.
- [42] Ruiz-Limón P, Ortega R, Arias de la Rosa I, Abalos-Aguilera M del C, Perez-Sanchez C, Jimenez-Gomez Y, et al. Tocilizumab improves the

- proatherothrombotic profile of rheumatoid arthritis patients modulating endothelial dysfunction, NETosis, and inflammation. *Transl Res* 2017;183:87–103. doi:10.1016/j.trsl.2016.12.003.
- [43] Kahlenberg J. Michelle , M.D., Ph.D.a and David A. Fox M. Advances in the Medical Treatment of Rheumatoid Arthritis. *NIH Public Access* 2011;27:11–20. doi:10.1016/j.hcl.2010.09.002.Advances.
- [44] Crofford LJ. Use of NSAIDs in treating patients with arthritis. *Arthritis Res Ther* 2013;15 Suppl 3:S2. doi:10.1186/ar4174.
- [45] Burmester GR, Pope JE. Novel treatment strategies in rheumatoid arthritis. *Lancet* 2017;389:2338–48. doi:10.1016/S0140-6736(17)31491-5.
- [46] Crofford L. Prostanoid biology and its therapeutic targeting. In Kelley's Textbook of Rheumatology. Crofford L: Prostanoid biology and its therapeutic targeting. In Kelley's Textbook of Rheumatology. 9th ed. 2013.
- [47] Mahmud T, Rafi SS, Scott DL, Wrigglesworth JM, Bjarnason I. Nonsteroidal antiinflammatory drugs and uncoupling of mitochondrial oxidative phosphorylation. *Arthritis Rheum* 1996;39:1998–2003.
- [48] Amin AR, Vyas P, Attur M, Leszczynska-Piziak J, Patel IR, Weissmann G, et al. The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. *Proc Natl Acad Sci U S A* 1995;92:7926–30.
- [49] Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994;265:956–9.
- [50] Díaz-González F, González-Alvaro I, Campanero MR, Mollinedo F, del Pozo MA, Muñoz C, et al. Prevention of in vitro neutrophil-endothelial attachment through shedding of L-selectin by nonsteroidal antiinflammatory drugs. *J Clin*

- Invest 1995;95:1756–65. doi:10.1172/JCI117853.
- [51] Díaz-González F, Sánchez-Madrid F. Inhibition of leukocyte adhesion: an alternative mechanism of action for anti-inflammatory drugs. *Immunol Today* 1998;19:169–72.
- [52] Gomez-Gavira MV, González-Alvaro I, Domínguez-Jiménez C, Peschon J, Black RA, Sánchez-Madrid F, et al. Structure-Function Relationship and Role of Tumor Necrosis Factor- α -converting Enzyme in the Down-regulation of L-selectin by Non-steroidal Anti-inflammatory Drugs. *J Biol Chem* 2002;277:38212–21. doi:10.1074/jbc.M205142200.
- [53] Herrera-García A, Domínguez-Luis M, Arce-Franco M, López-Fernández J, Feria M, Barreiro O, et al. In vivo modulation of the inflammatory response by nonsteroidal antiinflammatory drug-related compounds that trigger L -selectin shedding. *Eur J Immunol* 2013;43:55–64. doi:10.1002/eji.201242805.
- [54] Pillinger MH, Capodici C, Rosenthal P, Kheterpal N, Hanft S, Philips MR, et al. Modes of action of aspirin-like drugs: salicylates inhibit erk activation and integrin-dependent neutrophil adhesion. *Proc Natl Acad Sci U S A* 1998;95:14540–5.
- [55] Bertolotto M, Contini P, Ottonello L, Pende A, Dallegri F, Montecucco F. Neutrophil migration towards C5a and CXCL8 is prevented by non-steroidal anti-inflammatory drugs via inhibition of different pathways. *Br J Pharmacol* 2014;171:3376–93. doi:10.1111/bph.12670.
- [56] Kaplan HB, Edelson HS, Korchak HM, Given WP, Abramson S, Weissmann G. Effects of non-steroidal anti-inflammatory agents on human neutrophil functions in vitro and in vivo. *Biochem Pharmacol* 1984;33:371–8.

- [57] Ottonello L, Barbera P, Dapino P, Sacchetti C, Dallegri F. Chemoattractant-induced release of elastase by lipopolysaccharide (LPS)-primed neutrophils; inhibitory effect of the anti-inflammatory drug nimesulide. *Clin Exp Immunol* 1997;110:139–43.
- [58] Derouet M, Thomas L, Moulding DA, Akgul C, Cross A, Moots RJ, et al. Sodium salicylate promotes neutrophil apoptosis by stimulating caspase-dependent turnover of Mcl-1. *J Immunol (Baltimore, Md 1950)* 2006;176:957–65. doi:10.4049/jimmunol.176.2.957.
- [59] Laponi MJ, Carestia A, Landoni VI, Rivadeneyra L, Etulain J, Negrotto S, et al. Regulation of neutrophil extracellular trap formation by anti-inflammatory drugs. *J Pharmacol Exp Ther* 2013;345:430–7. doi:10.1124/jpet.112.202879.
- [60] Maria de Hair, Nicole IJff JJ and J van L. Long-Term Adverse Events after Daily Concomitant Treatment with 10mg Prednisone in the 2-Year Computer Assisted Management in Early Rheumatoid Arthritis Trial-II. *Arthritis Rheumatol* 2015;67.
- [61] Nakagawa M, Terashima T, D'yachkova Y, Bondy GP, Hogg JC, van Eeden SF. Glucocorticoid-induced granulocytosis: contribution of marrow release and demargination of intravascular granulocytes. *Circulation* 1998;98:2307–13.
- [62] Hirasawa N, Watanabe M, Mue S, Watanabe K, Tsurufuji S, Ohuchi K. Induction of neutrophil infiltration by rat chemotactic cytokine (CINC) and its inhibition by dexamethasone in rats. *Inflammation* 1992;16:187–96.
- [63] Salak JL, McGlone JJ, Lyte M. Effects of in vitro adrenocorticotrophic hormone, cortisol and human recombinant interleukin-2 on porcine neutrophil migration and luminol-dependent chemiluminescence. *Vet Immunol Immunopathol*

- 1993;39:327–37.
- [64] Llewellyn-Jones CG, Hill SL, Stockley RA. Effect of fluticasone propionate on neutrophil chemotaxis, superoxide generation, and extracellular proteolytic activity in vitro. *Thorax* 1994;49:207–12.
- [65] Yoshida N, Yoshikawa T, Nakamura Y, Takenaka S, Sakamoto K, Manabe H, et al. Methylprednisolone inhibits neutrophil-endothelial cell interactions induced by interleukin-1beta under flow conditions. *Life Sci* 1997;60:2341–7.
- [66] Schleimer RP, Freeland HS, Peters SP, Brown KE, Derse CP. An assessment of the effects of glucocorticoids on degranulation, chemotaxis, binding to vascular endothelium and formation of leukotriene B4 by purified human neutrophils. *J Pharmacol Exp Ther* 1989;250:598–605.
- [67] Filep JG, Delalandre A, Payette Y, Földes-Filep E. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. *Circulation* 1997;96:295–301.
- [68] Trowald-Wigh G, Håkansson L, Johannisson A, Edqvist LE. The effect of prednisolone on canine neutrophil function: in vivo and in vitro studies. *Acta Vet Scand* 1998;39:201–13.
- [69] Cox G, Austin RC. Dexamethasone-induced suppression of apoptosis in human neutrophils requires continuous stimulation of new protein synthesis. *J Leukoc Biol* 1997;61:224–30.
- [70] Nittoh T, Fujimori H, Kozumi Y, Ishihara K, Mue S, Ohuchi K. Effects of glucocorticoids on apoptosis of infiltrated eosinophils and neutrophils in rats. *Eur J Pharmacol* 1998;354:73–81.
- [71] Tsuji C, Shioya S. In vivo effect of methylprednisolone on lipopolysaccharide-

- induced superoxide production by pulmonary and circulating blood neutrophils in rats. *Circ Shock* 1994;42:128–34.
- [72] Ruiz LM, Bedoya G, Salazar J, García de O D, Patiño PJ. Dexamethasone inhibits apoptosis of human neutrophils induced by reactive oxygen species. *Inflammation* 2002;26:215–22.
- [73] Youssef P, Roberts-Thomson P, Ahern M, Smith M. Pulse methylprednisolone in rheumatoid arthritis: effects on peripheral blood and synovial fluid neutrophil surface phenotype. *J Rheumatol* 1995;22:2065–71.
- [74] Youssef PP, Cormack J, Evill CA, Peter DT, Roberts-Thomson PJ, Ahern MJ, et al. Neutrophil trafficking into inflamed joints in patients with rheumatoid arthritis, and the effects of methylprednisolone. *Arthritis Rheum* 1996;39:216–25.
- [75] Youssef PP, Triantafillou S, Parker A, Coleman M, Roberts-Thomson PJ, Ahern MJ, et al. Effects of pulse methylprednisolone on cell adhesion molecules in the synovial membrane in rheumatoid arthritis. Reduced E-selectin and intercellular adhesion molecule 1 expression. *Arthritis Rheum* 1996;39:1970–9.
- [76] Saag KG, Teng GG, Patkar NM, Anuntiyo J, Finney C, Curtis JR, et al. American College of Rheumatology 2008 recommendations for the use of nonbiologic and biologic disease-modifying antirheumatic drugs in rheumatoid arthritis. *Arthritis Rheum* 2008;59:762–84. doi:10.1002/art.23721.
- [77] Chatzidionysiou K, Emamikia S, Nam J, Ramiro S, Smolen J, van der Heijde D, et al. Efficacy of glucocorticoids, conventional and targeted synthetic disease-modifying antirheumatic drugs: a systematic literature review informing the 2016 update of the EULAR recommendations for the management of rheumatoid

- arthritis. *Ann Rheum Dis* 2017;76:1102–7. doi:10.1136/annrheumdis-2016-210711.
- [78] Chon WJ, Josephson MA. Leflunomide in renal transplantation. *Expert Rev Clin Immunol* 2011;7:273–81. doi:10.1586/eci.11.20.
- [79] Jung YH, Moon KC, Ha JW, Kim S-J, Ha I-S, Cheong HI, et al. Leflunomide therapy for BK virus allograft nephropathy after pediatric kidney transplantation. *Pediatr Transplant* 2013;17:E50–4. doi:10.1111/ptr.12029.
- [80] Teschner S, Burst V. Leflunomide: a drug with a potential beyond rheumatology. *Immunotherapy* 2010;2:637–50. doi:10.2217/imt.10.52.
- [81] Bilger A, Plowshay J, Ma S, Nawandar D, Barlow EA, Romero-Masters JC, et al. Leflunomide/teriflunomide inhibit Epstein-barr virus (EBV)- induced lymphoproliferative disease and lytic viral replication. *Oncotarget* 2017;8:44266–80. doi:10.18632/oncotarget.17863.
- [82] Cherwinski HM, McCarley D, Schatzman R, Devens B, Ransom JT. The immunosuppressant leflunomide inhibits lymphocyte progression through cell cycle by a novel mechanism. *J Pharmacol Exp Ther* 1995;272:460–8.
- [83] Xu X, Shen J, Mall JW, Myers JA, Huang W, Blinder L, et al. In vitro and in vivo antitumor activity of a novel immunomodulatory drug, leflunomide: mechanisms of action. *Biochem Pharmacol* 1999;58:1405–13.
- [84] Bartlett RR, Anagnostopoulos H, Zielinski T, Mattar T, Schleyerbach R. Effects of leflunomide on immune responses and models of inflammation. *Springer Semin Immunopathol* 1993;14:381–94.
- [85] Zielinski T, Müller H, Schleyerbach R, Bartlett RR. Differential effects of leflunomide on leukocytes : Inhibition of rat in vivo adhesion and human in vitro

- oxidative burst without affecting surface marker modulation 1994;278:276–8.
- [86] Montesinos MC, Desai A, Delano D, Chen J-F, Fink JS, Jacobson MA, et al. Adenosine A_{2A} or A₃ receptors are required for inhibition of inflammation by methotrexate and its analog MX-68. *Arthritis Rheum* 2003;48:240–7. doi:10.1002/art.10712.
- [87] Majumdar S, Aggarwal BB. Methotrexate suppresses NF-kappaB activation through inhibition of IkappaBalpha phosphorylation and degradation. *J Immunol* 2001;167:2911–20.
- [88] Cronstein BN, Naime D, Ostad E. The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. *J Clin Invest* 1993;92:2675–82. doi:10.1172/JCI116884.
- [89] O’Callaghan JW, Forrest MJ, Brooks PM. Inhibition of neutrophil chemotaxis in methotrexate-treated rheumatoid arthritis patients. *Rheumatol Int* 1988;8:41–5. doi:10.1007/BF00541349.
- [90] Kraan MC, de Koster BM, Elferink JG, Post WJ, Breedveld FC, Tak PP. Inhibition of neutrophil migration soon after initiation of treatment with leflunomide or methotrexate in patients with rheumatoid arthritis: findings in a prospective, randomized, double-blind clinical trial in fifteen patients. *Arthritis Rheum* 2000;43:1488–95. doi:10.1002/1529-0131(200007)43:7<1488::AID-ANR11>3.0.CO;2-G.
- [91] Weinmann P, Moura R a, Caetano-Lopes JR, Pereira P a, Canhão H, Queiroz M V, et al. Delayed neutrophil apoptosis in very early rheumatoid arthritis patients is abrogated by methotrexate therapy. *Clin Exp Rheumatol* 2007;25:885–7.

- [92] Ottonello L, Frumento G, Arduino N, Bertolotto M, Mancini M, Sottofattori E, et al. Delayed neutrophil apoptosis induced by synovial fluid in rheumatoid arthritis: role of cytokines, estrogens, and adenosine. *Ann N Y Acad Sci* 2002;966:226–31.
- [93] Raza K, Scheel-Toellner D, Lee C-Y, Pilling D, Curnow SJ, Falciani F, et al. Synovial fluid leukocyte apoptosis is inhibited in patients with very early rheumatoid arthritis. *Arthritis Res Ther* 2006;8:R120. doi:10.1186/ar2009.
- [94] Novaes GS, Mello SB, Laurindo IM, Cossermelli W. Low dose methotrexate decreases intraarticular prostaglandin and interleukin 1 levels in antigen induced arthritis in rabbits. *J Rheumatol* 1996;23:2092–7.
- [95] Sperling RI, Benincaso AI, Anderson RJ, Coblyn JS, Austen KF, Weinblatt ME. Acute and chronic suppression of leukotriene B4 synthesis ex vivo in neutrophils from patients with rheumatoid arthritis beginning treatment with methotrexate. *Arthritis Rheum* 1992;35:376–84.
- [96] Dewas C, Dang PM-C, Gougerot-Pocidalo M-A, El-Benna J. TNF-alpha induces phosphorylation of p47(phox) in human neutrophils: partial phosphorylation of p47phox is a common event of priming of human neutrophils by TNF-alpha and granulocyte-macrophage colony-stimulating factor. *J Immunol* 2003;171:4392–8.
- [97] Fujishima S, Hoffman AR, Vu T, Kim KJ, Zheng H, Daniel D, et al. Regulation of neutrophil interleukin 8 gene expression and protein secretion by LPS, TNF-alpha, and IL-1 beta. *J Cell Physiol* 1993;154:478–85. doi:10.1002/jcp.1041540305.
- [98] Cross A, Moots RJ, Edwards SW. The dual effects of TNFalpha on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1.

- Blood 2008;111:878–84. doi:10.1182/blood-2007-05-087833.
- [99] Ginis I, Tauber AI. Activation mechanisms of adherent human neutrophils. *Blood* 1990;76:1233–9.
- [100] Taylor PC, Peters AM, Paleolog E, Chapman PT, Elliott MJ, McCloskey R, et al. Reduction of chemokine levels and leukocyte traffic to joints by tumor necrosis factor α blockade in patients with rheumatoid arthritis. *Arthritis Rheum* 2000;43:38–47. doi:10.1002/1529-0131(200001)43:1<38::AID-ANR6>3.0.CO;2-L.
- [101] Verri WA, Souto FO, Vieira SM, Almeida SCL, Fukada SY, Xu D, et al. IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy. *Ann Rheum Dis* 2010;69:1697–703. doi:10.1136/ard.2009.122655.
- [102] Dominical VM, Bértolo MB, Almeida CB, Garrido VT, Miguel LI, Costa FF, et al. Neutrophils of Rheumatoid Arthritis Patients on Anti-TNF- α Therapy and in Disease Remission Present Reduced Adhesive Functions in Association with Decreased Circulating Neutrophil-Attractant Chemokine Levels. *Scand J Immunol* 2011;73:309–18. doi:10.1111/j.1365-3083.2011.02503.x.
- [103] Wittkowski H, Foell D, Klint E a., Rycke LD, Keyser FD, Frosch M, et al. Effects of intra-articular corticosteroids and anti-TNF therapy on neutrophil activation in rheumatoid arthritis. *Ann Rheum Dis* 2007;66:1020–5. doi:10.1136/ard.2006.061507.
- [104] den Broeder AA, Wanten GJA, Oyen WJG, Naber T, van Riel PLCM, Barrera P. Neutrophil migration and production of reactive oxygen species during treatment with a fully human anti-tumor necrosis factor-alpha monoclonal antibody in patients with rheumatoid arthritis. *J Rheumatol* 2003;30:232–7.

- [105] European Medicines Agency. Orenzia (abatacept): EU summary of product characteristics. 2016. n.d.
http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000701/human_med_000958.jsp&mid=WC0b01ac058001d124 (accessed November 15, 2017).
- [106] Hodge JA, Kawabata TT, Krishnaswami S, Clark JD, Telliez JB, Dowty ME, et al. The mechanism of action of tofacitinib - an oral Janus kinase inhibitor for the treatment of rheumatoid arthritis. *Clin Exp Rheumatol* 2016;34:318–28.
- [107] Maeshima K, Yamaoka K, Kubo S, Nakano K, Iwata S, Saito K, et al. The JAK inhibitor tofacitinib regulates synovitis through inhibition of interferon- γ and interleukin-17 production by human CD4⁺ T cells. *Arthritis Rheum* 2012;64:1790–8. doi:10.1002/art.34329.
- [108] van Vollenhoven RF, Fleischmann R, Cohen S, Lee EB, García Mejjide JA, Wagner S, et al. Tofacitinib or Adalimumab versus Placebo in Rheumatoid Arthritis. *N Engl J Med* 2012;367:508–19. doi:10.1056/NEJMoa1112072.
- [109] Genovese MC, Kawabata T, Soma K, Menon S, Clark JD, Hodge J, et al. Reversibility of pharmacodynamic effects after short-and long-term treatment with tofacitinib in patients with rheumatoid arthritis. *Arthritis Rheum* 2013;65:S193.
- [110] Burmester GR, Blanco R, Charles-Schoeman C, Wollenhaupt J, Zerbini C, Benda B, et al. Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitors: a randomised phase 3 trial. *Lancet* 2013;381:451–60. doi:10.1016/S0140-6736(12)61424-X.

- [111] Fleischmann R, Kremer J, Cush J, Schulze-Koops H, Connell CA, Bradley JD, et al. Placebo-Controlled Trial of Tofacitinib Monotherapy in Rheumatoid Arthritis. *N Engl J Med* 2012;367:495–507. doi:10.1056/NEJMoa1109071.
- [112] Cohen S, Radominski SC, Gomez-Reino JJ, Wang L, Krishnaswami S, Wood SP, et al. Analysis of Infections and All-Cause Mortality in Phase II, Phase III, and Long-Term Extension Studies of Tofacitinib in Patients With Rheumatoid Arthritis. *Arthritis Rheumatol* 2014;66:2924–37. doi:10.1002/art.38779.
- [113] Mitchell TS, Moots RJ, Wright HL. Janus kinase inhibitors prevent migration of rheumatoid arthritis neutrophils towards interleukin-8, but do not inhibit priming of the respiratory burst or reactive oxygen species production. *Clin Exp Immunol* 2017;189:250–8. doi:10.1111/cei.12970.
- [114] Ahmadzadeh N, Shingu M, Nobunaga M, Tawara T. Relationship between leukotriene B4 and immunological parameters in rheumatoid synovial fluids. *Inflammation* 1991;15:497–503.
- [115] Gürsel T, Firat S, Ercan ZS. Increased serum leukotriene B4 level in the active stage of rheumatoid arthritis in children. *Prostaglandins Leukot Essent Fatty Acids* 1997;56:205–7.
- [116] Chen M, Lam BK, Kanaoka Y, Nigrovic PA, Audoly LP, Austen KF, et al. Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. *J Exp Med* 2006;203:837–42. doi:10.1084/jem.20052371.
- [117] Shao W-H, Del Prete A, Bock CB, Haribabu B. Targeted disruption of leukotriene B4 receptors BLT1 and BLT2: a critical role for BLT1 in collagen-induced arthritis in mice. *J Immunol* 2006;176:6254–61.
- [118] Kim ND, Chou RC, Seung E, Tager AM, Luster AD. A unique requirement for

- the leukotriene B4 receptor BLT1 for neutrophil recruitment in inflammatory arthritis. *J Exp Med* 2006;203:829–35. doi:10.1084/jem.20052349.
- [119] Griffiths RJ, Pettipher ER, Koch K, Farrell CA, Breslow R, Conklyn MJ, et al. Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis. *Proc Natl Acad Sci U S A* 1995;92:517–21.
- [120] Kuwabara K, Yasui K, Jyoyama H, Maruyama T, Fleisch JH, Hori Y. Effects of the second-generation leukotriene B(4) receptor antagonist, LY293111Na, on leukocyte infiltration and collagen-induced arthritis in mice. *Eur J Pharmacol* 2000;402:275–85.
- [121] Weinblatt ME, Kremer JM, Coblyn JS, Helfgott S, Maier AL, Petrillo G, et al. Zileuton, a 5-lipoxygenase inhibitor in rheumatoid arthritis. *J Rheumatol* 1992;19:1537–41.
- [122] Awni WM, Braeckman RA, Granneman GR, Witt G, Dubé LM. Pharmacokinetics and pharmacodynamics of zileuton after oral administration of single and multiple dose regimens of zileuton 600mg in healthy volunteers. *Clin Pharmacokinet* 1995;29 Suppl 2:22–33.
- [123] Joshi EM, Heasley BH, Chordia MD, Macdonald TL. In vitro metabolism of 2-acetylbenzothiophene: relevance to zileuton hepatotoxicity. *Chem Res Toxicol* 2004;17:137–43. doi:10.1021/tx0341409.
- [124] Masferrer JL, Zweifel BS, Hardy M, Anderson GD, Dufield D, Cortes-Burgos L, et al. Pharmacology of PF-4191834, a Novel, Selective Non-Redox 5-Lipoxygenase Inhibitor Effective in Inflammation and Pain. *J Pharmacol Exp Ther* 2010;334:294–301. doi:10.1124/jpet.110.166967.
- [125] Lin H-C, Lin T-H, Wu M-Y, Chiu Y-C, Tang C-H, Hour M-J, et al. 5-

- Lipoxygenase inhibitors attenuate TNF- α -induced inflammation in human synovial fibroblasts. *PLoS One* 2014;9:e107890. doi:10.1371/journal.pone.0107890.
- [126] Uberti AF, Olivera-Severo D, Wassermann GE, Scopel-Guerra A, Moraes JA, Barcellos-de-Souza P, et al. Pro-inflammatory properties and neutrophil activation by *Helicobacter pylori* urease. *Toxicon* 2013;69:240–9. doi:10.1016/j.toxicon.2013.02.009.
- [127] Rzodkiewicz P, Gąsińska E, Gajewski M, Bujalska-Zadrożny M, Szukiewicz D, Maśliński S. Esculetin reduces leukotriene B4 level in plasma of rats with adjuvant-induced arthritis. *Reumatologia* 2016;54:161–4. doi:10.5114/reum.2016.62469.
- [128] Soto-Sánchez J, Santos-Juanes J, Coto-Segura P, Coto E, Díaz M, Rodríguez I, et al. Genetic variation at the CCR5/CCR2 gene cluster and risk of psoriasis and psoriatic arthritis. *Cytokine* 2010;50:114–6. doi:10.1016/j.cyto.2010.01.006.
- [129] Gong JH, Ratkay LG, Waterfield JD, Clark-Lewis I. An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. *J Exp Med* 1997;186:131–7.
- [130] Brühl H, Cihak J, Schneider MA, Plachý J, Rupp T, Wenzel I, et al. Dual role of CCR2 during initiation and progression of collagen-induced arthritis: evidence for regulatory activity of CCR2+ T cells. *J Immunol* 2004;172:890–8.
- [131] Hartl D, Krauss-Etschmann S, Koller B, Hordijk PL, Kuijpers TW, Hoffmann F, et al. Infiltrated neutrophils acquire novel chemokine receptor expression and chemokine responsiveness in chronic inflammatory lung diseases. *J Immunol* 2008;181:8053–67.

- [132] Stankovic A, Slavic V, Stamenkovic B, Kamenov B, Bojanovic M, Mitrovic DR. Serum and synovial fluid concentrations of CCL2 (MCP-1) chemokine in patients suffering rheumatoid arthritis and osteoarthritis reflect disease activity. *Bratisl Lek Listy* 2009;110:641–6.
- [133] Haringman JJ, Gerlag DM, Smeets TJM, Baeten D, van den Bosch F, Bresnihan B, et al. A randomized controlled trial with an anti-CCL2 (anti-monocyte chemoattractant protein 1) monoclonal antibody in patients with rheumatoid arthritis. *Arthritis Rheum* 2006;54:2387–92. doi:10.1002/art.21975.
- [134] Vergunst CE, Gerlag DM, Lopatinskaya L, Klareskog L, Smith MD, van den Bosch F, et al. Modulation of CCR2 in rheumatoid arthritis: A double-blind, randomized, placebo-controlled clinical trial. *Arthritis Rheum* 2008;58:1931–9. doi:10.1002/art.23591.
- [135] Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Annu Rev Immunol* 1997;15:675–705. doi:10.1146/annurev.immunol.15.1.675.
- [136] Grespan R, Fukada SY, Lemos HP, Vieira SM, Napimoga MH, Teixeira MM, et al. CXCR2-specific chemokines mediate leukotriene B₄-dependent recruitment of neutrophils to inflamed joints in mice with antigen-induced arthritis. *Arthritis Rheum* 2008;58:2030–40. doi:10.1002/art.23597.
- [137] Barsante MM, Cunha TM, Allegretti M, Cattani F, Policani F, Bizzarri C, et al. Blockade of the chemokine receptor CXCR2 ameliorates adjuvant-induced arthritis in rats. *Br J Pharmacol* 2008;153:992–1002. doi:10.1038/sj.bjp.0707462.
- [138] Coelho FM, Pinho V, Amaral FA, Sachs D, Costa V V., Rodrigues DH, et al. The chemokine receptors CXCR1/CXCR2 modulate antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature. *Arthritis*

- Rheum 2008;58:2329–37. doi:10.1002/art.23622.
- [139] Min S-H, Wang Y, Gonsiorek W, Anilkumar G, Kozlowski J, Lundell D, et al. Pharmacological targeting reveals distinct roles for CXCR2/CXCR1 and CCR2 in a mouse model of arthritis. *Biochem Biophys Res Commun* 2010;391:1080–6. doi:10.1016/j.bbrc.2009.12.025.
- [140] Blazek K, Eames HL, Weiss M, Byrne AJ, Perocheau D, Pease JE, et al. IFN- λ resolves inflammation via suppression of neutrophil infiltration and IL-1 β production. *J Exp Med* 2015;212:845–53. doi:10.1084/jem.20140995.
- [141] Kotenko S V., Gallagher G, Baurin V V., Lewis-Antes A, Shen M, Shah NK, et al. IFN- λ s mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003;4:69–77. doi:10.1038/ni875.
- [142] Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003;4:63–8. doi:10.1038/ni873.
- [143] Mennechet FJD, Uzé G. Interferon-lambda-treated dendritic cells specifically induce proliferation of FOXP3-expressing suppressor T cells. *Blood* 2006;107:4417–23. doi:10.1182/blood-2005-10-4129.
- [144] Koltsida O, Hausding M, Stavropoulos A, Koch S, Tzelepis G, Ubel C, et al. IL-28A (IFN- λ 2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease. *EMBO Mol Med* 2011;3:348–61. doi:10.1002/emmm.201100142.
- [145] Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. *Clin Exp Immunol* 2007;149:217–25. doi:10.1111/j.1365-2249.2007.03441.x.

- [146] Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;23:479–90. doi:10.1016/j.immuni.2005.09.015.
- [147] Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, et al. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol* 2008;181:4780–90.
- [148] Leung BP, Xu D, Culshaw S, McInnes IB, Liew FY. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. *J Immunol* 2004;173:145–50.
- [149] Verri WA, Guerrero ATG, Fukada SY, Valerio DA, Cunha TM, Xu D, et al. IL-33 mediates antigen-induced cutaneous and articular hypernociception in mice. *Proc Natl Acad Sci U S A* 2008;105:2723–8. doi:10.1073/pnas.0712116105.
- [150] Verri WA, Souto FO, Vieira SM, Almeida SCL, Fukada SY, Xu D, et al. IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy. *Ann Rheum Dis* 2010;69:1697–703. doi:10.1136/ard.2009.122655.
- [151] Lefrancais E, Roga S, Gautier V, Gonzalez-de-Peredo A, Monsarrat B, Girard J-P, et al. IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc Natl Acad Sci* 2012;109:1673–8. doi:10.1073/pnas.1115884109.
- [152] Sharma P, Azebi S, England P, Christensen T, Møller-Larsen A, Petersen T, et al. Citrullination of histone H3 interferes with HP1-mediated transcriptional repression. *PLoS Genet* 2012;8:e1002934. doi:10.1371/journal.pgen.1002934.
- [153] Ghari F, Quirke A-M, Munro S, Kawalkowska J, Picaud S, McGouran J, et al. Citrullination-acetylation interplay guides E2F-1 activity during the

- inflammatory response. *Sci Adv* 2016;2:e1501257. doi:10.1126/sciadv.1501257.
- [154] Kawalkowska J, Quirke A-M, Ghari F, Davis S, Subramanian V, Thompson PR, et al. Abrogation of collagen-induced arthritis by a peptidyl arginine deiminase inhibitor is associated with modulation of T cell-mediated immune responses. *Sci Rep* 2016;6:26430. doi:10.1038/srep26430.
- [155] Jang B, Kim HW, Kim J-S, Kim WS, Lee BR, Kim S, et al. Peptidylarginine deiminase inhibition impairs Toll-like receptor agonist-induced functional maturation of dendritic cells, resulting in the loss of T cell-proliferative capacity: a partial mechanism with therapeutic potential in inflammatory settings. *J Leukoc Biol* 2015;97:351–62. doi:10.1189/jlb.3A0314-142RR.
- [156] Seri Y, Shoda H, Suzuki A, Matsumoto I, Sumida T, Fujio K, et al. Peptidylarginine deiminase type 4 deficiency reduced arthritis severity in a glucose-6-phosphate isomerase-induced arthritis model. *Sci Rep* 2015;5:13041. doi:10.1038/srep13041.
- [157] Suzuki A, Yamada R, Chang X, Tokuhiko S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003;34:395–402. doi:10.1038/ng1206.
- [158] Suzuki A, Kochi Y, Shoda H, Seri Y, Fujio K, Sawada T, et al. Decreased severity of experimental autoimmune arthritis in peptidylarginine deiminase type 4 knockout mice. *BMC Musculoskelet Disord* 2016;17:205. doi:10.1186/s12891-016-1055-2.
- [159] Neeli I, Khan SN, Radic M. Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol* 2008;180:1895–902.

- [160] Wang Y, Li M, Stadler S, Correll S, Li P, Wang D, et al. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol* 2009;184:205–13. doi:10.1083/jcb.200806072.
- [161] Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs Are a Source of Citrullinated Autoantigens and Stimulate Inflammatory Responses in Rheumatoid Arthritis. *Sci Transl Med* 2013;5:178ra40-178ra40. doi:10.1126/scitranslmed.3005580.
- [162] Willis VC, Banda NK, Cordova KN, Chandra PE, Robinson WH, Cooper DC, et al. Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced arthritis. *Clin Exp Immunol* 2017;188:263–74. doi:10.1111/cei.12932.

Table 1. Effects of different therapeutic agents on neutrophils number and functions

Pharmacologic Agent	Effect on Neutrophils	Reference
----------------------------	------------------------------	------------------

ACCEPTED MANUSCRIPT

ROS, indicates reactive oxygen species; PG-2, prostaglandin E2; LTB4, Leukotriene B4; 5-LO, 5-lipoxygenase, NETs, neutrophils extracellular traps.

NSAIDs	Inhibit the adhesion process, prevent neutrophils chemotaxis and migration, decrease degranulation, elastase and ROS production, and promote apoptosis	[9, 56-59, 61-64]
Glucocorticoids	Increase the number of circulating neutrophils, inhibit chemotaxis, decrease the adherence capacity, delay apoptosis in circulating neutrophils, reduce the production of ROS, degranulation, and the release of inflammatory mediators	[67-73, 75-81]
Leflunomide	Reduces adhesion and oxidative burst, decreases migration into affected joints	[93]
Methotrexate	Inhibits chemotaxis, induces apoptosis of circulating neutrophils, in animal models decreases the intra-articular levels of PGE2 and thromboxane B2, downregulates the synthesis of LTB4 and inhibits 5-LO	[100,101,105,106]
Anti-TNF- α	Decrease the expression of adhesion molecules, inhibit chemotaxis, reduce the production of chemokines, and the generation of NETs	[44, 111-115]
Tocilizumab	Decreases the absolute neutrophils count, reduces oxidative burst and ROS generation, inhibits NETs formation, and downregulates the production of key inflammatory	[66, 69, 76, 77]

	mediators	
Rituximab	Decreases the absolute neutrophils count (not common)	[77]
Tofacitinib	Decreases the absolute neutrophils count, exerts anti-apoptotic effects, prevents migration	[114, 116, 117]

Table 2. New potential targets on neutrophils for the treatment of Rheumatoid Arthritis.

LTB₄, Leukotriene B₄; 5-LO, 5-lipoxygenase; CCR2, C-C motif chemokine receptor 2; CXCR1/2, CXC chemokine receptors 1 and 2; IFNLR1-IL28RA, IFN-receptor 1 (IFNLR1)–IL-28 receptor; IL-33, interleukin-33; PAD4, peptidylarginine deiminase 4

NEUTROPHIL TARGET	FUNCTION	FINDINGS	MODEL	REFERENCES
LTB ₄	Chemoattractant	LB ₄ levels are increased in the serum of RA patients, correlating with activity disease	Human	[128, 129]
		Neutrophil LB ₄ is required for inflammatory arthritis	Mouse	[130]
		Knockdown of LB ₄ receptors, BLT1/BLT2 protected from disease development	Mouse	[131]

		Neutrophil expression of LTB4 receptor, BLT1, was required for arthritis generation	Mouse	[132]
5-LO	Synthesis of LTB4	Zileuton improved clinical variables in RA patients, efficacy and medical acceptance was compromised	Human	[135-137]
		A 5-LO inhibitor (PF-4191834) effective in preclinical model of	Rat	[138]

		inflammation and pain		
		PF-4191834 reduced inflammation through TNF α pathwa y inhibition	Synovial fibroblast from RA patients	[125]
		Esculetin reduced neutrophil infiltration and inflammation	Mouse, Human neutrophil s	[139]
		Esculetin, a potent inhibitor of lipoxygenases, is effective in arthritis	Rat	[140]
CCR2	Inflammation,	Anti-CCL2 monoclonal antibodies prevent the	Mouse	[142]

	migration and adhesion	onset of arthritis		
		CCR2 blockade ameliorated activity RA disease	Mouse	[143]
		Peripheral blood and infiltrated RA neutrophils express high levels of CCR2	Human	[24, 144]
		Anti-CCL2 monoclonal antibody had no beneficial effects in RA patients (Phase II trial)	Human	[146, 147]
		Peripheral RA neutrophils express high levels of CCL2	Human	[31]
	Neutrophil	An oral		[150]

CXCR1/CXCR2	activation and accumulation	inhibitor of CXCR1/CXCR2, DF2162, significantly ameliorated arthritis	Rat	
		DF2162 decreased the influx of neutrophils into the joint	Mouse	[151]
		SHC563705 decreased clinical disease score, reducing inflammation and cartilage damage	Mouse	[152]
IFNLR1-IL28RA	Recruitment and migratory capacity	IFN γ 2-IL28A reversed the development of arthritis, through the reduction of the	Mouse	[153]

		migratory and inflammatory capacity on neutrophils.		
IL-33	Neutrophil migration and accumulation	Soluble IL-33R inhibited the development of arthritis	Mouse	[157]
		IL-33 contributed to the development of local inflammation	Mouse	[158]
		IL-33 induces neutrophil chemotaxis and it is reduced by therapies	Mouse Human neutrophils	[159]
PAD4	Inflammation,	PAD4 is critical for NETs formation	Human neutrophils	[168, 169]
		Extracellular		

	NET formation	PAD4 released by neutrophils increases cell activation, cytokine production and perpetuates inflammation.	Human neutrophils	[170]
		Knockout Pad4 mice has reduced number of neutrophils	Mouse	[165]
		PAD inhibition decreased the clinical score of arthritis	Mouse	[167]
		GSK199, a selective inhibitor of PAD4 prevented clinical and histological disease severity	Mouse	[172]

Figure 1. Pathogenic mechanisms of neutrophils in RA. In the RA setting, neutrophils express diverse chemokine receptors (BLT1, CXCR2, CCR1 and CCR2), release high levels of a number of inflammatory mediators (TNF- α , IL-1 β , IL-6, IL-8, IL-17 β , IL-20, IL-22, LTB4 and CXCL5) and express anti-apoptotic proteins (Mcl-1, G-CSF, GM-CSF, IFN γ and IL-15). These molecules act by increasing the migratory capacity of neutrophils, which results in the massive infiltration to the synovium, and prolonging cell survival within the joint. Under TNF- α activation, RA neutrophils can also express BlyS which is a potent activator of B-lymphocytes. The inflammatory cytokines alongside the autoantibodies are also able to induce NETosis in neutrophils (N) and LDGs at both the systemic and synovial levels. NETs containing numerous enzymes (MPO, NE and NADPH oxidase) and citrullinated proteins, as well as PADs, represent a source of new autoantibody generation that will activate B-cells to produce new autoantibodies. In addition, NETs can induce the expression of IL-8 and IL-6 in fibroblasts. Activated fibroblasts in RA joints also produce Cyr61, that mediates the expression of IL-8. These cytokines, in turn can induce the infiltration of neutrophils to the synovium and the formations of NETs, thus promoting bone damage.

RA indicates rheumatoid arthritis; TNF- α , tumor necrosis factor α ; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; IL-8, interleukin 8; IL-17 β , interleukin 17 β ; IL-20, interleukin 20; IL-22, interleukin 22; LTB4, leukotriene B4; CXCL5, chemokine (C-X-C motif) ligand 5; BLT1, leukotriene B4 receptor; CXCR2, IL8 receptor B; CCR1, CC chemokine receptor type 1; CCR2, CC chemokine receptor type 2; BlyS, B-lymphocyte stimulator; LDGs, low-density granulocytes; Mcl-1, Induced myeloid leukemia cell differentiation protein; G-CSF, granulocyte-colony stimulating factor; GM-CSF, Granulocyte-Macrophage colony stimulating factor, IFN γ interferon γ ; IL-15, interleukin 15; ACPAs, anti-citrullinated protein antigens antibodies; RF,

rheumatoid factor; NADPH, reduced nicotine adenine dinucleotide phosphate; ROS, reactive oxygen species; PAD, peptidyl arginine deiminase; NE, neutrophil elastase; MPO, myeloperoxidase; NETs, neutrophil extracellular traps.

ACCEPTED MANUSCRIPT

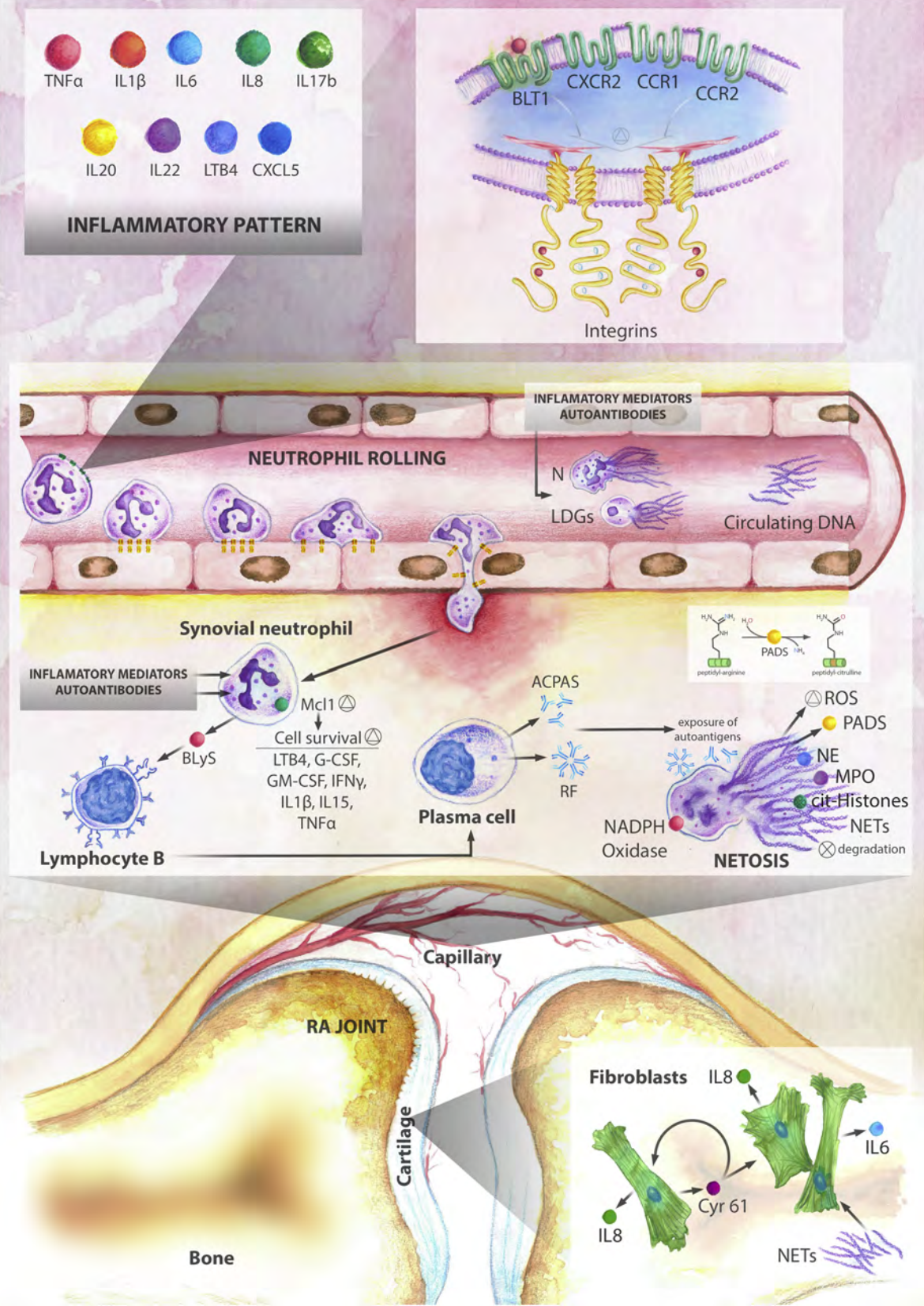


Figure 1