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

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

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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 theonset 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 considerablecontribution 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 ofneutrophils 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 affectsthe joints directly, since the synovium is the first site of the inflammatory cascade. Synovitis occurs when leukocytes infiltrate the synovial compartment, causingthe 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 osteoclastogenesisthereby 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 IL1-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-kB 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 neutrophilsurvival, including leukotriene B4, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), IL-1β, 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 inductors 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-1α, 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 enhancingthe 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-1β□in the affected joints, also playsan important role in the pathogenesis of RA since high levels of this molecule have been found in the synovial tissue ofantigen-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 akey 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 itparticipates 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 reducesthe 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 greatergeneration of NETs [20,21].MPO triggersthe 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 (NO_2^-) [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

NETsare 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, animpairment 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 apotential 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 includesthe 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 relevantcontribution 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 -chooseat 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 neutrophilchemotaxis 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-κB [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 effectsof 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], delayapoptosis in circulating neutrophils [69][70], and reducethe 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 withTNF-α [59].

In the specific RA setting, low doses of prednisone donot 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 physio-pathologic 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 cornerstoneand the main starting therapy for RA.

Growing evidencesuggests 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-1β, thesuppression 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 arhthritis (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 ascompared 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 neutrophilinflammatory 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 canactually 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 intothe 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 neutrophilfunctions 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-provenprogression 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 theearly 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 useboth 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-cellproliferation 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 neutrophilcount [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 overallmodulation 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]. Tofacitinibinhibited the anti-apoptotic effect of GM-CSF and IFN-γ on neutrophils from HDs. In addition, after incubating Tofacitinib for 20 hours at thehighest 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 relyonly on TNF-α inhibition and autoantibody production and which could fillthe 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 testedfor 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 shownthat 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 theonset 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 chemokinereceptors 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-TNFα 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-λ1, -λ2, and -λ3 (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-λ2 [140].

Despite the use of a different receptor complex, IFNLR1–IL28RA activates similar signaling pathways to thoseof the type I IFN receptor [141] [142]. IFN-λs 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 producedproduced by neutrophils, but not byother 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 agonistsof IFNLR1-IL-28 can have potential as novel therapeutics, since treatment with IL-28A specifically targetsIL-1β-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 ismainly 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-kB[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 IFNγ [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-1β, 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 toneutrophils, 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]. PAD4 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 playersin 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 bDMARDsis tolowerthe 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.

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Figure legends

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-1β, IL-6, IL-8, IL-17β, IL-20, IL-22, LTB4 and CXCL5) and express anti-apoptotic proteins (Mcl-1, G-CSF, GM-CSF, IFNγ and IL-15). These molecules act by increasing the migratory capacity of neutrophils, which results in the massive infiltration to the synovium, and prolonging cellsurvival 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 autoantibodygeneration 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 jointsalso produce Cyr61, that mediates the expression of IL-8. These cytokines, in turncan 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-1β, interleukin 1β; IL-6, interleukin 6; IL-8, interleukin 8; IL-17β, interleukin 17β; 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, IFNγ, 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.