Review

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Abstract: Rheumatoid arthritis (RA) is a progressive and systemic autoimmune disease, characterized with a chronic inflammatory process, affecting the lining of the synovial joints, many body organs/systems and blood vessels. Its pathological hallmarks are hyperplasic synovium, bone erosion, and progressive join destruction. Rheumatoid arthritis affects over 20 million people with a worldwide prevalence of 0.5-1.0%, exhibiting gender, ethnic and geographical differences. The progressive disability severely impairs physical motion, quality of life, and is finally leading to a shortened life span. The pathogenesis of RA is a complex and still poorly understood process in which genetic and environment factors are principally associated. The current treatment mostly relies on conventional/non-biological disease modifying anti-rheumatic drugs (cDMARDs), analgesics, non-steroidal anti-inflammatory drugs, glucocorticoids, steroids, immunosuppresants and biologic DMARDs, which only control inflammation and pain. Along with the side effects (drug toxicity and intolerance), these anti-rheumatic drugs possess limited efficacy. Therefore, the discovery of novel multi-target therapeutics with no side effects that function as inhibitors of RA-linked signaling systems are in high demand and this is in interest in both, patients and clinicians. Plant-derived extracts, nutritional supplements, dietary medicine and molecules with anti-inflammatory activity represent promising adjuvant agents or alternatives for RA therapeutics. This review aims not only to discuss the basic features of RA pathogenesis, risk factors and signaling pathways, but highlights the research progress in pre-clinical RA in vitro and in vivo models revealing new avenues in management of the disease in terms of comprehensive multidisciplinary strategies originating from medicinal plants and plant-derived molecules.

Keywords: Rheumatoid arthritis; risk factors; pathogenesis; signaling pathways; medicinal plants; natural products; *in vitro* and *in vivo* models

1. Introduction

Rheumatoid arthritis (RA) is the most common progressive and systemic autoimmune disease, characterized with a chronic inflammatory process, which predominantly affects the lining of the

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synovial joints (articular damage), as well as, a variety of other body organs/systems and blood vessels (extra-articular manifestations) [1–3]. The pathological hallmarks of RA are defined by hyperplasic synovium, bone erosion, and progressive join destruction due to autoantibodies production towards immunoglobulin G (IgG, named rheumatoid factor (RF)) and citurllinated proteins (anti-citrullinated protein antibodies (ACPAs)), carbamylated proteins (anti-carbamylated protein antibodies (anti-CarP)) and lately acetylated proteins (anti-acetylated protein antibodies). [4–6]. Both, RF and ACPAs have approximately equal sensitivity and specificity to RA. The disease is also marked up by elevated levels of C-creative protein (CRP), and erythrocyte sedimentation rate (ESR) [7]. The most common serum prognostic biomarkers for RA are RF and ACPA, since their appearance is observed approximately 4.5 years prior to clinical onset of the disease. A relatively new potential biomarker for RA, revealing high specificity is the oncoprotein survivin detected in 50.7% of RA patients and only 5.6% in controls [8]. Nevertheless, 50-80% of RA patients harbor autoantibodies are seropositive, while other patients are seronegative for these auto-antibodies. For that reason to identify RA patients, a set of clinical diagnosis *e.g.* physical examination, clinical symptoms, antibodies present in the blood and imaging findings are necessary to be applied [9–11].

1.1. Epidemiology Overview and Global Prevalence

Although RA can occur at any time, it is mainly manifested in elderly population peaking between the ages of 40 to 70 [12]. Rheumatoid arthritis affects over 20 million people and its average worldwide prevalence is about 0.5-1.0%, exhibiting gender (prevalence of female to male is in ratio 3:1), ethnic and geographical differences, therefore significantly varying among different populations [7,12]. Along with joint damage, extra-articular multiple co-morbidities affect cardiovascular, endocrine, neurological, ocular and pulmonary systems, including hematologic, renal and hepatic disorders [13]. The progressive disability severely impairs physical motion and the quality of life, finally leading to a shortened life span [6], reporting mortality rates twice higher in patients with RA and this number is increasing [14]. Based on analyses for the period 1990-2020, it was reported that in 2020 the RA patients were 17.6 million worldwide and the age-standardized global prevalence rate increased 14.1% since 1990. The age-standardized death rate decreased with 23.8% from 1990 to 2020, while the disability-adjusted life-years (DALYs) increased with 76.4%. In spite of that, RA is still a key public health concern worldwide. The global burden of RA has increased during the past decades and will continue to increase, forecasting that 31.7 million individuals will be living with RA until 2050 [15]. Rheumatoid arthritis has a negative impact on the most productive and active years (30-50 years) of an individual's lifespan [16], severely affecting the quality of daily life, including high disability rates and potential loss of labor [17]. Socioeconomic surveys indicated that people with severe (60%) or moderate (48%) pain experience additional work obstacles compared with dose with mild (34%) or no (19%) pain and significant correlation was found between severity, pain, disability, and early retirement [18]. The major economic costs for RA patients are based on both healthcare expenditures and loss of productivity [19]. For example the average annual cost of medical care for RA depending on the medication may vary between $12 500 to $36 000, while the Rheumatoid Arthritis Support Network estimates that low productivity, absenteeism, and lost wages can cost $1,500 to $22,000 per year, per patient [20]. In addition to the joint, many advanced patients develop systemic and comorbid extra-joint diseases, such as hypertension, diabetes mellitus, ischemic heart disease, chronic obstructive pulmonary disease, asthma, tuberculosis, chronic liver disease, hypothyroidism, hyperthyroidism, active malignancy etc., that significantly reduces life expectancy [7,21].

The clinical manifestation of RA differs in its early and untreated/inadequately treated late stage. The typical symptoms for the early stage are fatigue, swollen and tender joints, redness, stiffness not only in the morning, but also after a period of inactivity. On the other hand, the late stage is characterized with more severe manifestations, such as Swan neck deformity, Ulnar deviation and subcutaneous nodules formation [6,22], also bone corrosion, muscle atrophy, synovitis invasion of articular cartilage, sub-cartilage bone erosion, damage to ligaments and tendons [17], which may

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finally result in a premature death [15]. Rheumatoid arthritis has a multi-synovial form, which primary clinical manifestation is the repeated and symmetrical multiple micro arthritis [17]. It is a form of polyarthritis due to its involvement of multiple joints (typically six or more), mainly affecting hand, wrists, knees, feet, metacarpophalangeal, proximal interphalangeal and metatarsophalangeal joints. Erosions of the feet and hands are observed radiographically [17,23]. The pathogenesis of RA is a complex and still poorly understood process in which genetic and environment factors are principally associated with the disease onset and progression [21,23]. Joint inflammation is being triggered and maintained by invasion and interaction between several types of immune cells, including neutrophils, antigen-presenting cells (B-cells, macrophages and dendritic cells), as well as, T-cells and fibroblast-like synoviocytes (FLS) [16,24]. These chain events contribute to the releasing of pro-inflammatory cytokines, such as interleukin-1 (IL-1), -6, -17, prostaglandins and tumor necrosis factor alpha (TNF-α), which mediators activate further additional signaling pathways, including the upregulation of cyclooxygenase-2 (COX-2), and matrix metalloproteinases (MMPs), Janus kinase-signal transducers and activators of transcription (induced by IL-6), and nuclear factor kappa-B (NF-κβ) pathway (induced by IL-1 and TNF-α) that continue to generate and accumulate inflammation [16,24]. The above indexed cells and cytokines pattern the RA microenvironment (consisting mainly of extracellular matrix (ECM) and stromal cells), where the generated inflammation creates hypoxic environment and subsequently triggers the generation of reactive oxygen species (ROS), angiogenesis and as a consequence the formed microvessels cause the synovial membrane to invade the cartilage surface and to form pannus, destroying the structure and function of bone and cartilage [25].

Rheumatoid arthritis is often regarded as incurable and currently no adequate treatment is available [6]. The current treatments aim to control inflammation and pain, contributing to patients' symptoms relief and therefore avoiding or minimizing joint destructive processes [7]. The current treatment of RA mostly relies on conventional/non-biological disease modifying anti-rheumatic drugs (cDMARDs), analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (GC), steroids, immunosuppresants and biologic DMARDs (bDMARDs) [7,12,26]. However, in most cases the drugs need to be accepted weeks or month before take any effect and may cause liver and bone marrow toxicity, certain malignance and/or opportunistic effects [12,27]. The patients are considered as "difficult to treat" or "refractory" to RA and although the introduction of targeted synthetic DMARDs, RA treatment is still a great therapeutic challenge and considerable part of patients remain symptomatic [28]. Along with the side effects, such as drug toxicity and intolerance, these anti-rheumatic drugs possess limited efficacy, therefore the discovery of novel multi-target therapeutics with no side effects are in high demand and this is in interest in both, patients and clinicians [12]. Plant-derived extracts, nutritional supplements, dietary medicine and molecules with anti-inflammatory activity represent promising adjuvant agents or alternatives for RA therapeutics [12]. The pathogenesis of RA is tightly related to many characterized signaling pathways and the research attention has focused on discovering plant-derived molecules that function as inhibitors of RA-linked signaling systems [17].

This review attempts to recapitulate first of all the dynamics of RA pathogenesis, highlighting the role of risk factors, effector cells involved, the role of metalloproteinases, oxidative and nitrosative stress, angiogenesis, cell migration and invasion, as well as, the production of cytokines and chemokines. Next, we discuss the existing conventional therapies for RA and their limitations. A summary of various signaling pathways involved in RA development has been performed outlining the efficacy and mechanism of plants and their phytoconstituents in RA management supported by preclinical data based on state-of-the-art *in vitro* and *in vivo* models. Finally, we analyze the existing theoretical and practical challenges, and unanswered questions in the field aiming to improve the RA therapy by natural products.

1.2. Risk factors

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Unlike osteoarthritis, RA does not develop due to age, but its onset and development is rather as a result of a combination between immune dysfunction, hereditary (genetic factors) and environmental factors. Because of the complexity, it has not been thoroughly clarified yet, therefore research into the genes, pathways, and pathogenic immune cell subsets in RA has advanced the understanding of mechanisms involved in pathogenesis [16,27]. The major differences between healthy and RA joint, as well as its process of establishment has been illustrated on Figure 1.

Genetic factors

The contribution of genetic predisposition is thought to be about 50 to 60%, which has the most significant impact on the vulnerability to RA [29]. Genetic studies have been successful in determining the heritable component of RA susceptibility and outcome, of which RA severity and response to treatment are important [30]. The presence or absence of RF and ACPAs classifies RA into seropositive and seronegative and differences between the risk factors involved exists. Tyrosine phosphatase non-receptor type 22 (PTPN22) risk alleles [31,32], human leukocyte antigen D-related (HLA-DR) alleles [33], and tumors necrosis factor-receptor associated factor 1 (TRAF1) and complement component 5 (TRAF1/C5) related genes are the main genetic factors associated with an ACPA-positive subtype [34], while interferon regulatory factor 5 (IRF-5) is confined to the ACPA-negative subtype [35]. Despite the lack of concrete evidence supporting a direct role in RA pathology, some genetic markers regulate immune responses and account for the variations in RA susceptibility [21]. The strongest genetic association with RA susceptibility is located at the HLA locus. This is a specific HLA class II gene also known as a major histocompatibility complex (MHC) loci, encoding MHC molecules that may contain the shared epitope [4]. Individuals carrying a single HLA allele, have a five-fold higher risk of developing RA than individuals without this gene [36]. The susceptibility and outcome of RA may be related to specific HLA-DR alleles, however, these alleles vary by ethnicity and geographic region [37,38]. The HLA-DRB1 gene constitutes the strongest genetic association linked to RA, and the allele associated with the disease is named as a shared epitope, which is a conserved sequence of five amino acids at positions 70-74 of the HLA-DRB1 gene [17] and this concept has been highly correlated with the ACPA-positive RA [30]. The shared epitope hypothesis indicates that some alleles with this conserved sequence are linked with the pathogenesis of RA since they allow antigen-presenting cells to incorrectly present their antigens to T cells, which results in T cell mediated autoimmune responses that directly contribute to the RA pathogenesis [39].

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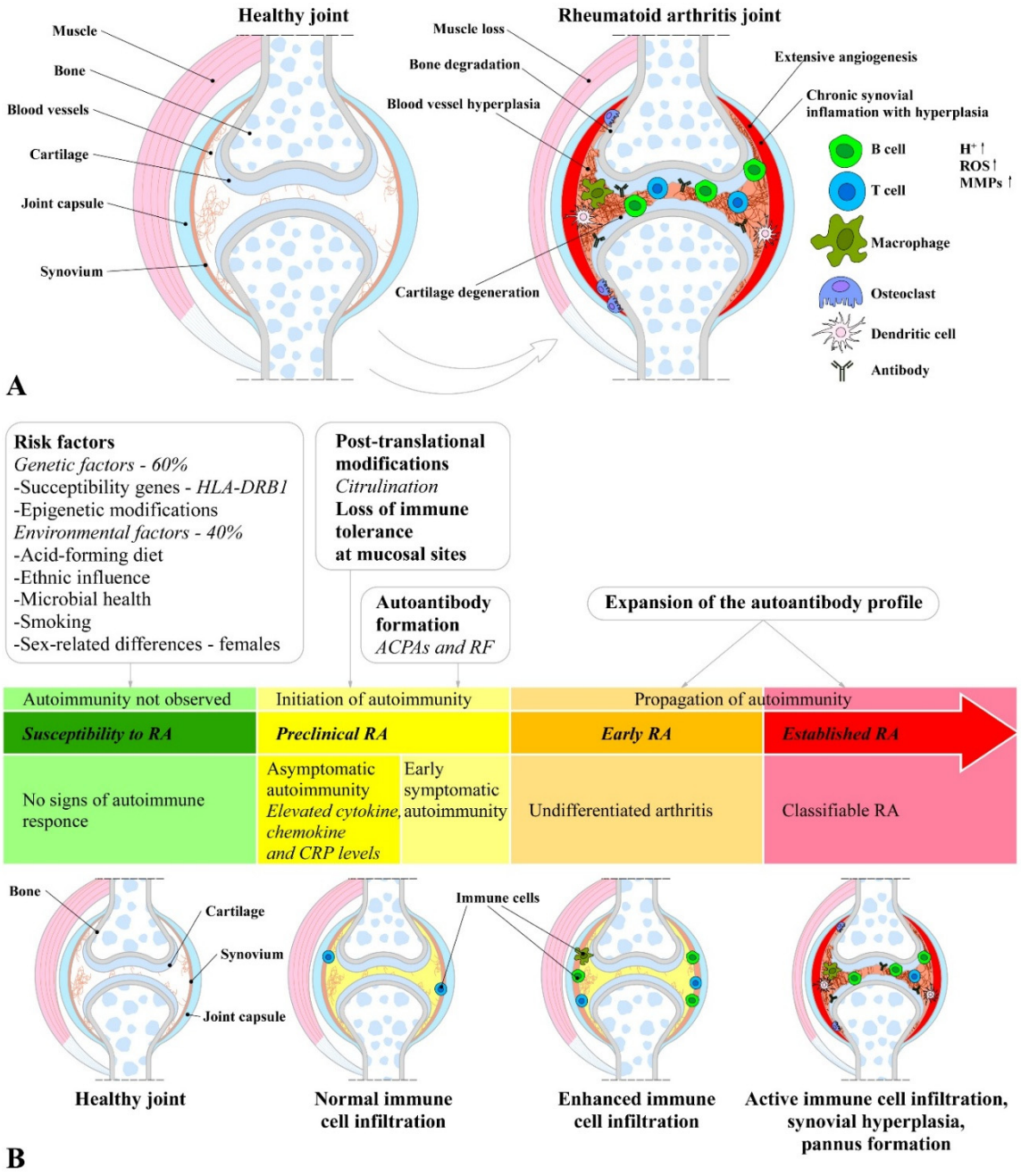

Figure 1. Comparison between healthy and RA joint and RA establishment. (**A**): due to immune activation, join swelling is observed reflecting in synovial inflammation, synovial hypertrophy (pannus) formation and osteoclast activation, extensive angiogenesis, bone and cartilage erosion, join space narrowing, joint structure destruction and muscle loss. Increased levels of metalloproteinases (MMPs) and reactive oxygen species (ROS) and decrease in pH is observed as well. The key players are accumulated cells of innate and adaptive immune systems, including T cells, dendritic cells, B cells, macrophages, and osteoclasts; Establishment of RA (**B**): multiple risk factors (genetic and non-genetic) are required to achieve the threshold for triggering RA. Before the first subclinical synovitis (inflammation of the synovium) and clinical symptoms, years before, the disease progression involves initiation and propagation of autoimmunity against modified self-proteins. Therefore, once established, RA can be classified according to the clinical symptoms.

On the other hand, single nucleotide polymorphisms (SNPs) are variations in the DNA code at a specific position in the genome and these variations have been associated with susceptibility to diseases, including RA [30]. A SNPs has been detected in T cells regulating the HLA-gene. In the presence of SNPs, the T cells induce tissue damage and inflammation linked to RA during the rapid

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resolution of the joint defects and therefore its detection may enable assessment of the risk of developing RA and hereditary autoimmune disorders in general [40]. The specific RA-related gene HLA-DR4 is found in 60% to 70% of patients diagnosed with RA, while it is detected in only 20% of the general population [41]. The HLA-DRB1 gene has been associated with the susceptibility of this disease, especially with the shared epitope coding alleles (HLA-DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1402, and *1001) [42]. In addition, genetic differences between ACPA-positive and ACPA-negative RA have been demonstrated, *e.g.* variants in *HLA-DRB1*, *PTPN22*, *BLK*, *Ankyrin Repeat Domain 55 (ANKRD55)* and *IL6ST* associate with RA regardless of serological status, whereas *AFF3*, *CD28* and *TNFAIP3* are found only in seropositive RA and *PRL* and *NFIA* are found only in seronegative RA [43,44]. All these variants demonstrate very well the RA susceptibility in both positive and negative type of RA. However, equally important is to identify markers of disease severity. In this regard it has been demonstrated that several markers for susceptibility are also associated with severity, *e.g. HLA-DRB1*, *IL2RA*, *DKK1*, *GRZB*, *MMP9* and *SPAG16* [43,45], although some of them, such as *FOXO3* are associated with severity alone [43,46]. There are also other HLA loci independently associated with RA susceptibility: amino acid position 9 of HLA-DPB1 (another HLA class II gene) [47] and two associations within HLA class I genes, such as amino acid position 9 of HLA-B [47] and position 77 of HLA-A [48]. Along with the HLA gene, there are many genetic variations outside the HLA complex, related to RA, such as *PTPN22*, *STAT4*, *TRAF1-C5*, *CTLA4* and *PADI4* [49]. The *PTPN22* gene encodes for the cytoplasmic lymphoid specific tyrosine phosphatase (Lyp). This enzyme has gained enormous interest due to a genetic SNP of its gene *PTPN22* rs2476601 (R620W) which has been associated with several human autoimmune diseases, including rheumatoid arthritis (RA). The Lyp has been shown to be a powerful inhibitor of T-cell and B-cell activation by binding to the SH3 domain of the Csk tyrosine kinase, an important negative regulator of T-cell and B-cell antigen receptor signaling [50–53]. Along with that, the role of Lyp in TNFα-induced priming of neutrophil ROS production has been investigated during RA development. It has been demonstrated that Lyp-selective inhibitors, inhibited TNFα-induced priming of neutrophil superoxide anion production, through inhibition of key pathways involved in neutrophil priming, such as inhibition of p47phox phosphorylation on Ser345, ERK1/2 phosphorylation and Pin1 [54].

Epigenetic factors

Genetic heterogeneity can not explain all aspects of RA, therefore examination of epigenetic factors and mechanisms might be of ultimate importance for the provision of novel therapeutic factors [55]. Epigenetics are heritable changes of gene expression without altering the DNA sequence, but epigenetics determines which genes are turned on and/or off, which mainly include histone modification, DNA methylation, and non-coding RNA mechanisms and these processes can be affected by different genetics and environmental factors. The epigenetic modifications are reversed processes, and the corresponding enzymes which control histone modification or DNA methylation could be proposed as drug targets for RA [17]. DNA methylation is the most commonly occurring postreplication DNA modification under the activity of DNA methyltransferases (DNMT), which transfers the methyl group from S-adenosine methionine (SAM) to the DNA sequence. DNA methylation occurs at the cytosine of CpG (cytosine-phosphoric acid guanine) islands to produce 5MC, most of which are located in the promoter region. Hypermethylation in the promoter region is related to gene silencing or gene inactivation, while its hypomethylation activates transcriptional activity and promotes gene expression of extracellular matrix proteins, growth factors/receptors, matrix-degrading enzymes, and adhesion molecules [56]. For example, several studies show that fibroblast-like synoviocytes (FLS) and peripheral blood mononuclear cells (PBMCs) in RA patients are characterized by extensively hypomethylated genomic DNA. Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) have a unique, non-random methylation pattern-methylome, which is specifically reorganized during the disease progression and varies depending on the joint localization [55], and DNA methylation reduction is often found in highly proliferative tissues and is associated with a relative lack of methyl groups' donor S-adenosylmethionine (SAM) [17]. T-box transcription factor 5 (TBX5) regulates expression of pro-inflammatory cytokines and chemokines in SF including

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CXCL12 (chemokine C-X-C motif ligand 12) chemokine. Upon hypomethylation *TBX5* increases its own expression and that of *CXCL12* which is associated with protein accumulation in RA patients and contributes to chronic inflammation [57]. The promoter demethylation of *IL-6* and *IL-10* genes in a single CpG sequence contribute to the increase in cytokine levels as the disease progresses [58]. Comprehensive analysis of DNA methylation in RA-SFs identified 1 859 differently methylated loci, of which the hypomethylated ones such as *CHI3L1*, *CASP1*, *STAT3*, *MAP3K5*, *MEFV*, and *WISP3* were of critical importance and found to be involved in cell migration, adhesion, transendothelial penetration and interactions in the extracellular matrix [59]. It has been reported that the PBMCs from RA patients have a significant overall DNA hypomethylation state compared to healthy people [60]. Analyses of the whole-genome DNA methylation and mRNA expression profiles of PBMCs from patients with RA revealed that approximately 1,046 DNA methylation sites were closely associated with the pathogenesis of RA. Among the identified differentially methylated positions (DMPs) and genes formed an interferon-inducible gene interaction network (such as *MX1*, *IFI44L*, *DTX3L* and *PARP9*). Methylation of PARP9 was correlated with mRNA level in Jurkat cells and T lymphocytes isolated from patients with RA. The *PARP9* gene exerted significant effects on Jurkat cells (eg, cell cycle, cell proliferation, cell activation and expression of inflammatory factor IL-2) [61]. Epigenetic modification in immune cells is also critical for the development of RA. The differentially methylated patterns of B lymphocytes in RA and systemic lupus erythematosus (SLE) patients and in the control group CpG sites were located in the *CD1C*, *TNFSF10*, *PARVG*, *NID1*, *DHRS12*, *ITPK1*, *ACSF3*, and *TNFRSF13C* genes [62]. On the other hand, hypermethylation of 4 CpG dinucleotides in exon 7 of *LRPAP1* has been correlated with patients who demonstrated no response to therapy by TNF inhibitors compared to responders. The *LRPAP1* gene is expressed in PBMCs and encodes the chaperone of low density lipoprotein receptor-related protein 1, that affects the activity of transforming growth factor beta (TGF-β) [63]. The aberrant function of regulatory T cells (Treg) in RA patients was associated with the hypermethylation of a specific region in the promoter of the cytotoxic T-lymphocyte associated protein 4 (CTLA-4;−658 CpG) in comparison with healthy controls. DNA hypermethylation prevents binding of the nuclear factor of activated T cells (NF-AT) with cytoplasmic one, called NF-ATc2, which leads to decrease of *CTLA-4* expression. As a consequence, Treg cells were unable to induce expression and activation of the tryptophan-degrading enzyme indoleamine 2.3-dioxygenase (IDO), which in turn resulted in a failure to activate the immunomodulatory kynurenine pathway [64]. Furthermore, treatment with methotrexate induced DNA hypomethylation of FoxP3 locus in Treg. This results in the gene upregulation with consequent increase of CTLA-4 concentration and normalization of Treg function in RA. These studies clearly illustrate how aberrant DNA methylation can affect cell functions and how epigenetic mechanisms can be used in therapy [65].

Histone modification is a post-translational modification of a specific site on histones in chromatin. Acetylation, methylation, phosphorylation, and ubiquitination are all included within the modifications of the histone tails and among them acetylation is the most common. Histone deacetylases (HDAC) have a significant role in the activation or silent regulation of pro-inflammatory genes, and their inhibitors are often used to study the pathogenesis of RA. The HDAC activity and histone H3 acetylation status in PBMCs are potential biomarkers for evaluating the disease activity [56]. For example, the hyperacetylation state caused by the decreased activity and expression of HDACs promotes the pro-inflammatory processes and ultimately leads to RA in PBMCs [66]. Mice with a T cell-specific deficiency of HDAC1 (HDAC1-cKO) were found to be resistant to the development of collagen-induced arthritis (CIA), while its activation produces pro-inflammatory factors. The HDAC1 is a promising target for the treatment of RA patients, since the inflammatory cytokines, IL-17 and IL-6, were significantly reduced in the serum of HDAC1-cKO mice. Along with that, selective HDAC inhibitors restrained chemokine receptor 6 (CCR6) upregulation in a mouse model and human $CD4^+$ T-cells [67]. Another potential target for RA treatment is SIRT1 (a type 3 histone deacetylase that possesses anti-inflammatory properties) and can reduce the inflammatory responses in RA by regulating M1/M2 macrophages polarization. For example, activated SIRT1

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promotes the phosphorylation of adenosine monophosphate-activated protein kinase α (AMPKα)/acetyl-CoA carboxylase in macrophages through upregulation of the M2 genes, such as *MDC*, *FcεRII*, *MrC1*, and *IL-10* expression. Simultaneously, activated SIRT1 downregulates the LPS/γ interferon mediated NF-κB activity by inhibiting p65 acetylation and M1 genes (including *CCL2*, *iNOS*, *IL-12p35*, and *IL-12p40*) expression [68].

MicroRNAs (miRNAs) widely present in all organisms and present endogenous non-coding single-stranded small RNAs of about 22 nucleotides in length [56]. Initially, the miRNA gene is transcribed to form a primary miRNA in the nucleus, cleaved by Drosha to form a precursor-miRNA. Exportin-5 transfers miRNA to the cytoplasm where it is cleaved by Dicer to form mature miRNA duplexes, which are then unfolded, and a miRNA strand is added to the RNA-induced silencing complex and act as a post-translational repressor of the gene [17]. MicroRNAs have been implicated in the occurrence and progression of many diseases, including RA. For example, hsa-miR-132-3p, hsa-miR-146a-5p, and hsa-miR-155-5p are potential biomarkers of responsiveness to methotrexate (MTX) therapy, which levels were lower in responders [69]. However, the downregulation of miR-10a in RA-FLs accelerated NF-κB activation and significantly promoted the production of various inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8 and MCP-1 and matrix metalloproteinase (MMP)-1 and MMP-13 [70]. Some miRNAs have been regulated in RA and it has been demonstrated that DNA methylation increased the expression of miR-203, which lead to increased secretion of MMP-1 and IL-6 via the NF-κB pathway and contributed to the activated phenotype of RA-FLs [71]. The upregulation of miR-203 induces RA through promoting the generation of MMP-1 and IL-6 [71]. Depletion of miR-19b regulates positively NF-κB signaling through suppression of a regulon of negative regulators of the same pathway [72].

Environmental factors

Many environmental factors, including smoking, alcohol, air pollution, and exposure to insecticides, viruses, bacteria, toxic minerals or mineral oil increase the risk of RA [21]. Most of the listed factors here support the hypothesis that the aetiopathogenesis of RA is of 'mucosal origin', in which the autoimmune processes that lead to the development of RA are triggered in the mucosa-associated lymphoid tissues in the lung, the oral cavity and the gut, prior to systemic spread [73]. Rheumatoid arthritis related autoantibodies may be generated in the lung mucosa and in draining lymph nodes prior to the onset of clinically apparent RA [74–76]. Smoking is the strongest environmental factor in RA development and raises the risk of RA in a gradient fashion, with double risk among smokers with a 20-year history of tobacco use compared with nonsmokers. The correlation between tobacco use and RA is strongest or almost limited to RF- or ACPA-positive individuals [77] with at least one copy of the shared epitope alleles HLA-DR Beta 1 [78] and has no or very little effect on ACPA-negative [79]. The risk for developing RA between shared epitope and smoking can be increased by 20-fold or more compared with nonsmokers who do not carry the shared epitope [80]. Actually, no association between passive smokers and risk of developing RA has been observed [81]. In addition, the increased risk associated with smoking might be mediated by epigenetic modifications, as smoking was significantly associated with hypomethylation of certain DNA regions [82]. Population-based studies revealed that smoking is a strong risk factor for RA in men rather in women, which effect might be due to hormonal differences in the modulation of the immune cascade activated by smoking [83]. Air pollution and the inhaled particulars, such as pulverized cement, silica, asbestos, glass fibers, textile dust and many others are all associated with RA development [84,85]. For example, there is correlation between silicosis and RA, which mainly affects patients with ACPA-positive RA and is caused due to silica dust inhalation [86]. Chronic exposure to silica can lead to rheumatoid pneumoconiosis, also known as Caplan's syndrome, a rare disease of RA patients who have developed silicosis [87].

Lifestyle factors (nutrition and gut microbiota)

It has been suggested that some healthy diets, *e.g.* Mediterranean diet has modest protective effect in individuals with seropositive RA [88]. The consumption of fruits and fish, including omega-3 fatty acids has a protective effect related to RA-associated autoimmunity [89–91]. Long-term

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supplementation of omega-3 and vitamin D resulted in a 25-30% lower incidence of RA [92]. Coffee consumption may be a risk factor for RA, a possible explanation being the involvement in the production of RF [93]. The intake of raw meat and sugar-sweetened sodas is associated with increased risk of RA [94–96]. Microbiota (periodontal and gut) and infection microorganisms are associated with increased risk of RA development as well [97]. For example the oral microbiota *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* causing periodontal disease, also related with RA development [98,99]. The dysregulation many intestinal bacteria are associated with RA onset. For example, expansion of *Prevotella* species is associated with RA-related autoimmunity and a marker for early disease preclinical development [100]. Other genus, such as *Bacteroides*, *Eggerthella* and *Collinsella* have also been related to RA [101–103]. Several infectious agents have been considered as possible causes of RA, including rubella virus, Epstein–Barr virus, and mycoplasma organisms [21].

Personal factors

Although both, males and females are at risk of RA development, RA is more prevalent in women with a with a female-to-male sex ratio ranging from 4:1 in younger individuals to less than 2:1 in older populations with the disease [100]. This might be due to hormonal changes (such as from contraceptive use) or a sudden decline of oestrogenic function (seen in menopause, or with the use of anti-oestrogenic therapies) [104,105]. The manifestations of RA improve or even disappear during pregnancy [106]. In women, RA most commonly becomes symptomatic around middle age or at the time of menopause. Men have a later disease onset, are more likely to be positive for RF and have higher titers of ACPAs [107]. Epidemiologic studies have shown a complex interaction between RA and obesity. Obesity would contribute to the development of seronegative RA, especially in younger women, although it is suggested a decreased risk of RA among men [108]. Adipose tissue secretes different pro-inflammatory and anti-inflammatory factors, including the adipokines leptin, adiponectin, resistin, and visfatin as well as cytokines and chemokines, such as TNF-α, IL-6, which participates in RA development [109].

1.3. Mechanisms and (pato)etiology of Rheumatoid Arthritis Initiation, Development and Progression

The pathogenesis of RA is a complex and poorly understood in terms of causes, initial triggers and progression process [16]. However, the initiation of RA involves an interplay among components of the innate and adaptive immune responses, where immune cells (T-cells, B-cells, mast cells, and dendritic cells) and synoviocytes (macrophages and fibroblasts) are the key players [8]. In general, the RA pathophysiology goes through three distinct stages: 1) occurrence of immune abnormalities; 2) synovial inflammation and extensive proliferation; and 3) joint deterioration and bone erosion [8]. The mechanisms involved in initiation and progression of RA are presented on Figure 2.

The initiation of RA has multifactorial origin including many genetic and environmental factors. Collectively, all these factors initiate the early development of RA, including post-translational modifications of a wide range of cellular (collagen) and nuclear (histones) proteins, including the conversion of the amino acid arginine to citrulline (a process called citrullination) through peptidyl arginine deiminase, type IV enzyme (PADI4; EC: 3.5.3.15) [1,110]. After citrullination or other post-translational modifications (acetylation or carbamylation) the altered modified self-proteins are recognized by immune system through binding to major histocompatibility complex (MHC) protein heterodimers, especially those containing the shared epitope and produce antigen presenting cells (APCs), such as dendritic cells and macrophages, which carry antigen to lymph node [4,22,111]. These APCs activate CD4 T-helper (Th) cells causing stimulation and proliferation of B-cells, which in turn distinguish into plasma cells and produce autoantibodies such as ACPA (targeting citrullinated proteins) and RF (targeting IgGs). The presence of these and other antibodies can be detected up to 10 years before the clinical disease onset [4,22,112]. The antibodies bind with its fixed complement to form immune complex and release chemotactic factors such as C5a and consequently due to chemotactic gradient, inflammatory cells are subsequently recruited to the rheumatoid joint where they are activated and cause localized inflammation [22,113]. The APCs such as plasmacytoid dendritic cells (PDC) and myeloid dendritic cell (MDC) in response to modified protein, express HLA

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class II molecules, cytokines (such as IL-12, -15, -18, and -23), and co-stimulatory molecules leading to activation of synovial T cells. Dendritic-cell–derived TGF-β and interleukins (IL-1β, -6, -21, and -23) supports Th17 cells (pathogenic cells) differentiation and suppress the differentiation of regulatory T cells (Treg) which shifts T-cell homeostasis toward inflammation. Activated Th17 cells release cytokines such as IL-17, IFN-γ to provoke expansion of the intimal lining and to recruit macrophages, which release interleukins (IL-1, -6, -12, -15, -18, and-23), TNF-α, TGF-β, granulocyte–macrophage colony-stimulating factor (GM-CSF), macrophage migration inhibitory factor (MIF), MMPs, disintegrin, ADAMTs, prostaglandins, leukotrienes, and reactive nitric oxide. They all together cause FLSs proliferation known as synovial hyperplasia [1,22,30]. Treg cells produce anti-inflammatory cytokines IL-10 and TGF-β and inhibit autoimmunity, therefore the balance between Th17 and Treg is important in the pathology of RA [114]. The increase in mass of synovial cells and immune cells in joints leads to pannus formation (thick, swollen synovial membrane with granulating tissue consisted of myofibroblast, fibroblast and inflammatory cells) [22,115].

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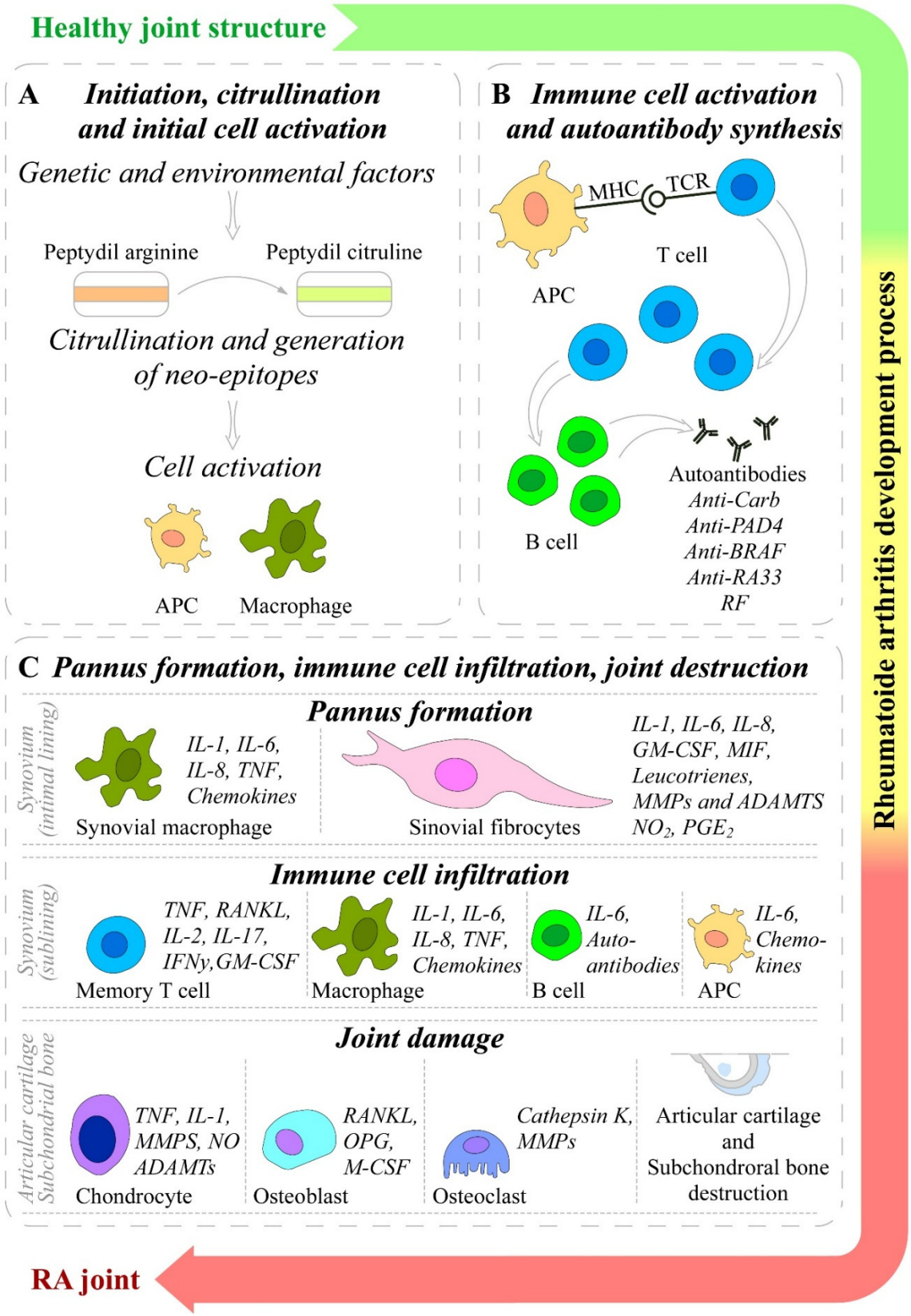

Figure 2. Mechanisms involved in initiation and progression of RA. (**A**): the onset of autoimmunity is supposed to occur in the mucosa (*e.g.* mouth, lung and gut) by the production of neo-epitopes resulting of post-translational modifications, such as by citrullination or carbamylation; (**B**): the altered peptides (neo-peptides) are recognized by antigen-presenting cells (APCs) of the adaptive immune system and (**C**): are presented to adaptive immune cells in lymphoid tissues activating an immune response, and induce autoantibody formation (*e.g.* ACPA and RF); (**C**): activated immune cells and complexes activate synovial cells, such as fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes of the intimal lining and APCs in the sublining area, thus

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producing a range of inflammatory factors and resulting in expansion and formation of the cartilage- and bone-invasive pannus. The activation and infiltration of immune cells (T cells, B cells and macrophages) of the sublining area further contribute to the excessive production of inflammatory factors, auto-antibodies, and synovial vascular leakage, resulting in articular cartilage and subchondral bone destruction due to matrix-degrading enzymes and a de-balanced bone homeostasis characterized by an imbalanced RANKL/RANK/OPG system and activated osteoclasts.

This hyperplastic pannus tissue contributes to cartilage damage but may also be responsible for propagation and systemic spreading of inflammation by migrating between joints or other organs [22,26]. The synovial immune cell infiltration transform the paucicellular synovium into chronically inflamed tissue [1]. Due to the resulting local hypoxia, new vessels are formed, which facilitate the inflammatory process by increasing the amount of adaptive immune cells, and especially CD4+ Th cells infiltrating the synovial sublining. Lymphocyte infiltrates accumulate and form aggregates, which may develop germinal centers that facilitate local T cell - B cell interactions. In these ectopic germinal structures are specific pathologic follicular helper T cells (Tfh), which promote B-cell responses and (auto)antibody production within pathologically-inflamed non-lymphoid tissues [1,116]. It has been established that specific pathogenic infiltrating immune cell subsets, such as IL-1β positive pro-inflammatory monocytes, autoimmune-associated B cells, and peripheral helper T (Tph) cells sharing similarities with Tfh cells, distinct subsets of CD8+ T cells, as well as mast cells, contribute to the inflammatory pattern of the RA synovial lining/sublining [117–122]. Finally, the invasive and destructive FLS-front of synovial tissue (called the pannus), attaches to the articular surface and contributes to local matrix destruction and cartilage degradation. The chondrocytes of the damaged articular cartilage contribute to the vicious cycle of cartilage degeneration by inducing inflammatory cytokines, such as IL-1β and TNF-α, as well as MMPs and nitric oxide (NO). Additionally, FLSs negatively affect the subchondral bone by activation and maturation of bone-resorbing osteoclasts. Osteoclasts are highly responsive to autoantibodies; pro-inflammatory cytokines, in particular TNF, IL-1, and IL-6; and more importantly, receptor activator of nuclear factor kappa B ligand (RANKL), which is the key regulator of osteoclastogenesis. RANKL binds to its receptor, the receptor activator of nuclear factor-B (RANK), and activates osteoclasts, leading to an enhancement of bone resorption and destruction. Conversely, osteoblasts that play a key role in the regulation of anabolic bone metabolism produce bone matrix constituents, induce bone matrix mineralization, and modulate osteoclasts through the production of osteoprotegerin (OPG) [32]. Although osteoblasts producing OPG, which is a decoy receptor for RANKL, results in protection from bone destruction by osteoclasts, they also generate RANKL and M-CSF, both of which contribute to osteoclastogenesis. Imbalanced bone remodeling both in the subchondral and periarticular bone of joints leads to bone erosions and periarticular osteopenia; generalized bone loss is a general feature of established RA [1,123].

Effectors Cells Involved in Rheumatoid Arthritis Pathology

Rheumatoid arthritis has a complex pathogenesis and the activation of cells of both, innate and adaptive immunity and aberrant cytokine production are the main events responsible for the generation and development of pathological immune response. Consequently, synovial inflammation leading to massive destruction of cartilage and bone structures is observed. The pathogenesis of RA involves a diverse array of effector cells, including T cells, B cells, macrophages, and fibroblasts, each contributing to the inflammatory milieu and tissue damage observed in affected individuals [124,125].

The association of the particular MHC-II alleles in RA chronic inflammation supported the role of CD4+ T-cells. Disease-associated HLA-DR alleles, and particularly those with the ”shaped epitope” may present arthritis-related peptides, such as those modified by citrullination, leading to the stimulation and expansion of autoantigen-specific CD4+ T cells in the joints and lymph nodes [126].

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As in the regular immune response, the autoimmune process starts with antigen presentation of the processed antigen on APCs, and triggering the activation of naïve T lymphocytes. This activation includes proliferation and secretion of cytokines as IL2, IFN-γ, TNF, and IL-4 that leads to modulation of the immune system through differentiation of various subsets of cells like T helper (Th)1, Th2, T follicular helper (Tfh), Th17 and regulatory T (Treg) cells. The Th1/Th2 imbalance is thought to exacerbate the inflammatory processes in RA, as a lack of Th2-mediated regulation allows for unchecked Th1-driven inflammation. Particularly CD4+ T helper cells and CD8+ cytotoxic T cells, play pivotal roles in RA pathology. A notable increase in the ratio of CD4+ to CD8+ T cells has been documented in the blood of RA patients, suggesting a skewed immune response favoring CD4+Tcell activation [127]. Furthermore, studies have shown that effector memory CD8+ Tcells are significantly altered in RA, with a decrease in their numbers in peripheral blood, potentially due to their migration to inflamed tissues [128]. This migration is indicative for their pathogenic role because they can produce pro-inflammatory cytokines such as IFN-γ, contributing to the inflammatory environment within the synovium [129].

Th17 cells are characterized by their production of IL17 and have emerged as critical player in RA pathology. These cells promote the recruitment of neutrophils and enhance the production of pro-inflammatory cytokines, contributing to joint inflammation and damage [130,131]. Studies have shown that Th17 cells are enriched in the synovial fluid of RA patients, correlating with disease severity and joint destruction [132,133]. The differentiation of Th17 cells is often favored in the inflammatory milieu of RA witch further perpetuates the cycle of inflammation and tissue damage [134]. T Follicular Helper (Tfh) cells are essential for B cells in the germinal centers, promoting their differentiation into plasma cells that produce of high-affinity antibodies. In RA, the frequency of circulating Tfh cells is significantly elevated, and these cells are associated with increased levels of autoantibodies, such as anti-citrullinated protein antibodies [135,136]. The presence of Tfh cells in the synovial tissue of RA patients suggests that they play a direct role in sustaining the autoimmune response and contributing to the chronicity of the disease.

Regulatory T (Treg) cells are typically involved in maintaining tolerance and preventing excessive inflammation. In the RA, their functional capacity is often compromised. This dysfunction leads to inadequate suppression of effector T cell responses, allowing for the persistence of inflammation and autoimmunity [137].

Macrophages are central to the inflammatory processes in RA, acting as a major producers of pro-inflammatory cytokines such as TNF-α and IL-1β. They are found in increased numbers within the synovial tissue, correlating with disease severity and joint erosion [138,139]. Additionally, macrophages expressing heparin-binding EGF-like growth factor (HB-EGF) have been shown to enhance fibroblast invasiveness, further exacerbating joint destruction [140,141].

B cells contribute significantly to RA through the production of autoantibodies and pro-inflammatory cytokines. Dysregulation of B cells subsets, including the expansion of RANKL+ effector B cells, has been observed in RA patients, indicating their involvement in osteoclastogenesis and joint destruction [142]. The efficacy of B cell depletion therapies, such as Rituximab, reinforces the pathogenic role of B cells in RA, as these cells not only produce antibodies but also act as antigen-presenting cells, perpetuating the inflammatory cycle [143]. Furthermore, the presence of FcRL4+ B cells in the synovial fluid suggests a unique subset that may contribute to the inflammatory process through enhanced activation and cytokine production [144].

Fibroblast-like synoviocytes are critical effector cells in RA, contributing to the formation of the invasive pannus that characterizes the disease. These cells are activated by various inflammatory mediators and exhibit increased production of matrix metalloproteinases (MMPs) and cytokines, facilitating tissue remodeling and joint destruction [145,146]. The interaction between FLSs and infiltrating immune cells, particularly macrophages, creates a feedback loop that sustains inflammation and joint damage [147,148]. Recent studies have identified functionally district subsets of FLSs that correlate with disease activity, suggesting that targeting these cells may offer therapeutic potential [145].

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2.1. Cytokines and the Impact on Effector Cells

Experimental studies on RA patients as well as on different mouse models point to the role of interactions between cytokines and effector cells in the disease progression [149,150]. Genetically modified TNF-α transgenic and IL-1Ra knockout mouse models of RA showed the importance of TNF-α and Th17 in the activation of the immune cells [151,152]. RA can also be induced and by the administration of different agents generating local or systemic inflammation. In the most frequently used model of RA - collagen-induced arthritis, the presence of collagen type II specific CD4+ T cells leads to the activation of collagen-specific B lymphocytes together with the secretion of various pro-inflammatory cytokines (TNF-α, IL-6), although the anti-inflammatory IL-10 and IL-1 receptor antagonist can also be detected in the affected tissues [150,153]. In antigen-induced model of RA, the antigen binds directly to the cartilage and induce inflammation with $CD4^{+}CCR6^{+}$ T cells via IL23 secretion [154]. Another mouse model of RA is the proteoglycan-induced arthritis in which scheduled injections of human cartilage proteoglycan resulted in synovial inflammation and infiltration of immune cells. CD4+ T cell immune response is observed with high levels of TNF-α, IL-1β, -6 and -12 in the experimental mice [155]. Finally, RA can be induced in various mouse strains by the administration of specific anti-collagen type II monoclonal antibodies. In this model the major effector cells appeared to be macrophages and neutrophils, although B- and T-lymphocytes also participated [156].

In RA patients, the role of effector immune cells and cytokine secretion has also been studied. Recent study reported that accumulation of B lymphocytes in synovium correlates positively with the severity of the disease [157]. B cells are the major source of autoantibodies to various antigens as some antibodies can be detected before the onset of the disease, whereas the presence of others indicates more severe form of RA [158]. In addition, being effective antigen-presenting cells [159] and producers of a number of pro-inflammatory cytokines [160,161], B lymphocytes stimulate autoreactive T-lymphocyte differentiation and proliferation [162]. RA pathogenesis is also caused by activated T cells and massive infiltration of CD4+ T lymphocytes can be observed in inflamed synovial tissues [157]. One particular population – peripheral helper PD-1+CD4+ T cells has been shown to have a central place in B cell co-stimulation and secretion of pro-inflammatory cytokines [163]. Although part of the innate immune system, another important players in RA pathogenesis are the neutrophils. Abnormal neutrophil activation due to aberrations in gene expression or in metabolic pathways, can lead to inappropriate local degranulation with subsequent chronic inflammation [164]. Finally, macrophages in synovium participate in the development of the inflammatory process by secretion of pro-inflammatory cytokines, chemokines and metabolites [165]. In addition, Zec et. al. have recently reported that synovial inflammation is initiated by lining macrophages in the niches and attract preferentially neutrophils in the inflamed tissues [166].

All these data demonstrated the complexity of RA pathogenesis and proposed interactions between the immune cells as well as particular cell populations as new targets for the forthcoming therapies.

2.2. The Role of Metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases belonging to the superfamily of metzincin proteases, involved in the extracellular matrix (ECM) degradation and remodeling. The MMPs have a critical role in RA, since they degrade ECM to destroy the integrity of synovial, cartilage and bone tissue through selectively cleavage of many non-matrix components present in the extracellular environment, such as cell surface receptor, cytokines, chemokines, cell-cell adhesion molecules, clotting factors and other proteinases like binding proteins to involve in inflammation and immunity in RA [167,168]. The key role of MMPs in immune cell development, function and inflammatory response turned them as attractive target therapy in RA disease [169]. Tissue inhibitors of metalloproteinases (TIMPs) are produced by all connective tissue cells and are firmly and irreversibly bound to the active MMPs and pro-MMPs to form a 1:1 non-selective complex to take part in ECM homeostasis [170]. These TIMPs control tissue breakdown by blocking the

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activities of MMPs and when imbalance occurs due to increased production of MMPs or a lack of TIMPs adequate regulation, the pathogenesis of RA is being initiated due to dysregulated tissue remodeling (the fine balance between ECM degradation and production) [171]. The ECM is typically composed of the interstitial connective tissue matrix and a basement membrane (BM). The major hydrolases responsible for tissue breakdown are MMP1, 8, 13 and membrane-type I metalloproteinase (MT1-MMP) and further MMP9 degrade type II collagen fragments to generate immunodominant epitope [172]. The MMPs 1 and 13 predominate in RA due to their capacity to restrict collagen degradation rate, while MMP13 is considered to have a dual role in RA since their capability of degrading aggrecan and proteoglycan, suggesting it has a dual role in ECM destruction in RA [173,174]. In RA mice model, during joint inflammation has been observed down-regulation of collagens (*COL1A1*, *COL3A1* and *COL5A1*) accompanied by increase in metalloproteases (*MMP3* and *MMP11*), and down-regulation of enzymes involved in matrix degradation, such as *ADAMTS1* and *ADAMTS2* (a disintegrin and metalloprotease with thrombospondin motifs) [175]. Similarly, in TNFα-treated decellularized chondrocyte matrix many different collagens (*COL1A1*, *COL1A2*, *COL2A1*, *COL5A1*, *COL5A2*, *COL12A1*, *COL14A1*), but also aggrecan and chondroadherin were strongly downregulated in the damaged matrix, while matrix-degrading enzymes including *MMP2*, *-3*, *-13* and *19*, as well as, *ADAMTS5* and *ADAMTSL4* were upregulated [176]. The MMPs also participate in bone destruction processes, cell migration and invasion, as well as, in cytokine and chemokine processing [177]. The MMPs participation in joint destruction has related through three mechanisms: 1) Adhesion to chondrocytes, leading to degradation of collagen and cartilage damage; 2) Provoking of imbalance in affected join homeostasis (through regulation of inflammatory cytokines and chemokines) and activation of inflammatory signaling pathways that promote osteoclast differentiation and bone resorption; 3) Promotion of cell migration and invasive angiogenesis [169]. The FLs are involved in both, synovial inflammation and bone erosion in RA through production of various factors such as TNFα, IL-1β, -6, -8, MMP-1, and MMP-13. Stimulated chondrocytes are able to express various proteases, including MMP-1, -2, -3, -7, -8, -9, -10, -13, -14, ADAM9, 10, 17 and ADAMTS4, all related to cartilage destruction [70,178]. Interleukin-1β is inducing a variety of MMPs, including MMP-1, -3, -8, -13, -14, -29 and activating osteoclasts to breakdown the cartilaginous matrix [168]. Bone destruction can be effectively ameliorated by inhibiting osteoclast differentiation and regulating multiple signaling pathways including osteoclast differentiation, IL-17, and TNF, as well as, MMP-9 and protein kinase B (AKT) signaling pathway [179]. In collagen induced arthritis (CIA) in mice bone destruction was correlated with the significantly elevated levels of *MMP-2* and *MMP-9* in serum, as well as, elevated levels of serum TNF-α, IL-6, and IL-1β [180]. In RA mice, the cartilage destruction process has been slowed down through downregulation of the expression of AKT1, VEGFA, IL-1β, IL-6, MMP-9, ICAM1, VCAM1, MMP-3, MMP-13 and TNF-α [181].

The destruction of the BM of synovial cells enable them to migrate through the tissue and permeate interior of the joint. It has been reported that MMP2 and MT1-MMP are essential for instigating invadopodia generation, cell migration and invasion [182]. In the CIA induced arthritis cartilage destruction and bone erosion was reduced by decreased protein levels of TNF-α , IL-1β, IL-6, iNOS, COX2, MMP-1, and MMP-3 in ankle joint tissue [183]. Inhibitors of epithelial-mesenchymal transition (EMT) significantly reduced the excessive proliferation, migration and invasive behavior of RA-FLs by reduction of MMP1, -3 and -13 secretion. In addition, the expression of N-Cadherin (an EMT marker) has been also reduced [184]. The RA-FLs migration and invasion mechanisms were significantly inhibited through inhibiting the classical TLR4-NF-κB inflammatory pathway and regulating the dynamic balance of MMP-2/TIMP-2, MMP-9/TIMP-1 [185]. Inhibition of the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling pathways (ERK/JNK) significantly attenuated the migration and invasion n RA-FLs through Leukocyte Ig-like receptor A3 (LILRA3), which resulted in decreased expression of expression of IL-6, IL-8 and MMP3 [186]. Similarly, the decreased expression of MMP-1 and -3, as well as, the pro-inflammatory factors

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(IL-1 and IL-6) and the elevated level of the pro-apoptotic factor (Bax nd cleaved caspase 3) inhibited the RA-FLs invasion *via* inactivation of WNT/β-catenin signaling pathway [187].

2.3. The Role of Angiogenesis

An intensive and important process in early stage of RA is angiogenesis (the development of new blood vessels), which is regulated by many inducers and inhibitors along with the involvement of pro-angiogenic factors like acidic and basic fibroblast growth factors (FGFs), transforming growth factor (TGF)-β, angiopoietin, placental growth factor, and vascular endothelial growth factor (VEGF) [188,189]. A previously unknown mechanism based on inhibition of the angiogenic functional module of circHIPK3/miR-149-5p/FOXO1/VEGF, demonstrated to have a significant protective effect on RA-FLS and CIA synovium, thus confirming the cross-talk between circular RNAs (circRNAs) and RA [190]. A broad investigation in RA patients revealed extensive correlation between pro-inflammatory and proangiogenic profile of the disease activity. The most important angiogenic markers were angiopoietin-1, neuropilin-1, Tie-2, endostatin, platelet factor 4 (CXCL4), interleukin-8 (IL-8, CXCL8), vascular cell adhesion molecule 1 (VCAM-1), VEGF and placenta growth factor (PlGF) [191]. Another mechanism involved in RA angiogenesis suppression is the hypoxia inducible factor-1α/ vascular endothelial growth factor/ angiopoietin 2 (HIF-1α/VEGF/ANG2) axis [192]. Interestingly, the release of heat shock protein 70 kDa (HSP70) can induce angiogenesis. However, the inhibited proteins in the sphingosine Kinases 1/sphingosine-1-phosphate/sphingosine-1-phosphate receptors/Gα protein subunit (SphK1/S1P/S1PRs/Gαi) pathway prevent HSP70 release and therefore producing anti-angiogenic effect [193].

2.4. The Role of Free Radicals

Free radicals and especially reactive oxygen species (ROS) play vital roles in RA pathogenesis and are key modulators of join inflammation [194]. When ROS are over-generated and/or the antioxidant system is in imbalance, ROS and its related metabolites are accumulated excessively and cause oxidative stress. Hypoxia and oxidative stress might be important drivers of the inflammatory processes in arthritic joints. Excessive ROS accumulation might contribute to somatic mitochondrial DNA (mtDNA) alterations, including mtDNA mutations in synovial tissue and somatic cells of RA patients and increased mitochondrial mutagenesis associated with reduced oxygen tension (pO2) in synovial membranes, which induced a pro-inflammatory mitochondrial phenotype [195–197]. It has been reported that ROS production increased lipid peroxidation, protein oxidation and DNA damage in the peripheral blood of RA patients [198,199]. Some markers of DNA damage, such as 8-Hydroxyl-2-deoxyguanosine (8-oxodG) has been found in synovial fluids and blood of RA patients [200].

3. Current Rheumatoid Arthritis Drug Treatment

The treatment for rheumatoid arthritis is focused on controlling inflammation and relieving pain with the end goal being low disease activity or remission. To achieve this, the European League Against Rheumatism (EULAR) has listed 10 recommendations for the management of RA with disease-modifying antirheumatic drugs (DMARDs). Furthermore, based on those recommendations an initiative called treat-to-target (T2T) was developed with the idea that RA treatment shouldn't be universal but rather individual for each patient [117,201].

The overarching principle of the T2T approach is the shared decision making between patient and rheumatologist on the next step in the treatment plan based on the efficacy of previous treatments and the treatment goals. Creating an individual therapeutic approach follows an algorithm adapted from the 2016 update on the EULAR recommendations. The treatment guideline is divided into 3 phases with each phase focusing on a different class of DMARDs based on the stage of the disease upon first diagnosis. Failure to achieve the treatment goal within 6 months of starting the therapy moves the patient to the next phase [202].

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3.1. Conventional Synthetic DMARDs (csDMARDs)

Glucocorticoids (GCs) are the most widely used anti-inflammatory drugs in the field of rheumatology. Intra-articular injections are used to treat acute disease flares, but this type of treatment has been associated with severe adverse reactions. To prevent this, low-dose oral GCs should be used only for short-term pain relief [203]. For long-term control of the inflammation, csDMARDs treatment should be started as soon as rheumatoid arthritis is diagnosed.

Methotrexate

Methotrexate (MTX, Trexall) is usually the first choice for RA treatment as it has been proven to be effective as low-dose monotherapy. Oral administration is usually starts at a dose of 15 mg/week but can be increased up to 25 mg. The mechanism of action of low-dose MTX is thought to be the inhibition of aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC). AICAR accumulates in the cell which leads to increased adenosine release (adenosine is a potent immunosuppressant) [204,205]. Common side effects include hematologic abnormalities, gastrointestinal problems, elevated liver enzymes, fatigue and nausea [206].

Leflunomide

Leflunomide (Arava) is administered orally 50 mg/week. Its effect is lymphocyte-specific immunomodulation. Leflunomide inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, which plays a key role in the *de novo* synthesis of the pyrimidine ribonucleotides which in term prevents clonal expansion of activated lymphocytes [207]. The most common adverse effects are diarrhoea, elevated liver enzymes, rashes, and hypertension [208].

Sulfasalazine

Sulfasalazine (SSZ, Azulfidine) was designed specifically for the treatment of rheumatoid arthritis. It combines an antibiotic, sulphapyridine, with an anti-inflammatory, salicylic acid, linked via an azo bond. The treatment of RA with SSZ is effective with the main effects being on the gut microbiota and inflammatory cell functions although the exact mechanism of action is not completely understood. Adverse reactions include gastrointestinal problems, rashes and nausea [209].

3.2. Biologic DMARDs (bDMARDs)

If the patient continues to display moderate disease activity without poor prognostic markers the therapy should be adjusted to a combination of csDMARDs instead of monotherapy. At the appearance of poor prognostic markers and failure to achieve low disease activity due to csDMARDs' lack of efficacy and/or toxicity the therapy should move forward to phase Ⅱ – using biologic DMARDs. Those agents are highly specific and target pathways of the immune system so screening, and treatment of any latent infections is highly advised before starting any bDMARDs [210].

Tumor Necrosis Factor-alpha inhibitors (TNFi)

TNF-α is a cytokine with both pro-inflammatory and immunoregulatory functions. In the context of rheumatoid arthritis, dysregulated TNF-α was the first cytokine to be proven to directly cause tissue destruction as well as lead to the overproduction of other pro-inflammatory cytokines. Hence, blocking TNF-α signalling is one of the most effective ways to slow down the disease progression [211,212].

Since 2000, five TNFi are available. Each antibody has different molecular structure, administration and dose but all have the same effect – binding soluble and/or membrane-bound TNF-α. This can be summarized in Table 1.

Table 1. Molecular structure, administration and dose of the five FDA approved TNFi antibodies [213].

Antibody	Molecular structure	Administration and dose
Infliximab (IFX, Remicade)	Chimeric IgG1 monoclonal antibody	Intravenous injections 3-10 mg/kg every 4-8 weeks

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Etanercept (ETN, Enbrel)	Recombinant human fusion protein (TNF-α receptor bound to Fc fragment)	Subcutaneous injections 50 mg/week or 25 mg/twice a week
Adalimumab (ADA, Humira)	Recombinant human IgG monoclonal antibody	Subcutaneous injections 25 mg/twice a week
Golimumab (GOL, Simponi)	Human IgG monoclonal antibody	Subcutaneous injections 50 mg/month
Certolizumab Pegol (CZP, Cimzia)	Recombinant humanized Fab fragment	Subcutaneous injections 400 mg at weeks 0, 2 and 4 followed by 200 mg/ every 2 weeks

Interleukin-1 inhibitor

Anakinra (Kineret) is a recombinant human IL-1 receptor antagonist administered subcutaneously 100 mg once a day. Studies on the effectiveness of Anakinra suggest that it might be dependent on the cytokine profile of the patient and that the drug works better in combination with other DMARDs [214,215].

Interleukin-6 receptor inhibitor

Besides TNF-α, IL-6 is the other cytokine proven to have a key role in the pathogenesis of rheumatoid arthritis. Tocilizumab (TCZ, Actemra) is a humanized recombinant IgG monoclonal antibody that binds to the soluble and membrane-bound IL-6 receptor. It's injected intravenously 8 mg/kg every 4 weeks. Studies have shown that Tocilizumab is effective and safe as monotherapy or in combination with other DMARDs with both short-term and long-term effects regardless of the stage of the disease [216,217].

Anti-CD20 antibody

CD20 is a membrane-bound molecule on the surface of B-cells, which plays a role in their development and differentiation into plasma cells. Rituximab (RTX, Rituxan) is a chimeric monoclonal antibody originally created to bind to the CD20 molecule on the surface of peripheral B-cells in blood cancer patients but since then has been proven effective for treating RA by depleting B-cells in the synovium as well. Administration is intravenous, two 1000 mg doses 2 weeks apart [218,219].

3.3. Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells, also known as multipotent mesenchymal stromal cells, are a heterogeneous group of cells with a plastic-adherent, fibroblast-like morphology. *In vitro* they can differentiate into osteocytes, chondrocytes, and adipocytes. According to the criteria established by the International Society for Cellular Therapy (ISCT), MSCs are identified based on the expression of surface markers such as CD73, CD90, CD105, CD44, CD71, and CD106, while lacking markers associated with hematopoietic and endothelial cells, including CD34, CD45, CD11b, CD14, and CD31 [220,221].

The MSCs' main role in physiology is supporting the function and maintenance of hematopoietic stem cells (HSCs) within the bone marrow. From MSCs, the structural elements of the specialized HSC microenvironment are formed; those are referred to as the "niche," which is essential for regulating HSC behaviour, including their self-renewal, differentiation, and survival. Through direct cell-to-cell interactions and the secretion of various cytokines, growth factors, and extracellular matrix components MSCs modulate the balance between hematopoietic stem cell quiescence and activation, ensuring a steady supply of blood cells while preventing exhaustion of the stem cell pool [222,223].

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The MSCs' therapeutic potential in autoimmune diseases and inflammatory disorders is due to their critical role in suppressing T-cell proliferation. Several mechanisms for MSC-mediated inhibition of proliferation have been described, primarily secreting immunosuppressive cytokines like TGF-β, IL-10, which induce a state of energy or functional inactivity in T cells. In addition, MSCs express surface molecules such as PD-L1, which bind PD-1 receptors on T cells, promoting T-cell apoptosis [224,225]. Lastly, it has been described that MSCs also influence the polarization of T cells, promoting the expansion of regulatory T cells, which further supress the immune response and maintain immune tolerance [226].

Preclinical studies in animal models of rheumatoid arthritis have demonstrated that MSCs can effectively reduce inflammation, inhibit immune activation, and promote tissue repair in damaged joints. MSCs migrate to inflamed tissues where they secrete both anti-inflammatory cytokines, such as TGF-β and IL-10, and pro-inflammatory cytokines, such as TNF-α and IL-6. In models with acute joint damage, MSCs have been shown to differentiate into chondrocytes and osteoblasts, restoring the structural integrity to the affected joints, decreasing bone erosion, and reducing cartilage degeneration [227,228].

A key focus of all preclinical studies is to understand the mechanisms behind MSC homing in inflamed tissues and their interaction with the immune system. Research has been done to identify factors that influence the effectiveness of MSC therapy, including the source of MSCs (e.g., bone marrow, adipose tissue, or umbilical cord), culture conditions, and delivery methods (e.g., intra-articular injection or systemic administration) [229].

4. In Vitro Studies Using Plant-Derived Natural Products for the Management of Rheumatoid Arthritis and Signaling Pathways

Multiple transcription factors and signaling pathways are involved in the pathogenesis of RA, the most important and key pathways are the MAPK (mitogen-activated protein kinase), NF-κB (nuclear factor kappa B), PI3/AKT (phosphatidylinositol 3 kinase-AKT, also known as PKB), JAK/STAT (Janus-activated kinase signal transduction and activator of transcription), Wnt/β-catenin (Wingless/Integrated), SYK/BTK (spleen tyrosine kinase)/Bruton's tyrosine kinase), and Notch [16,17]. The major signaling pathways and their possible modulation by plant-derived molecules has been presented on Figure 3.

The NF-κB signaling pathway controls many biological processes, mainly inflammation that is associated with RA. Inflammation has been intensively observed in the early and late stage of RA and is triggered by NF-κB activation, in both T cells and antigens- presenting cells directly or indirectly by extracellular and/or intracellular stimuli (*e.g*. IL-1β, -6, TNF-α, MMPs *etc*.). The NF-κB signaling may be activated by two different ways: direct activation (canonical and noncanonical pathways) mediated by inhibitor of kappa B (IkB) kinase (IKK) and NF-kB-inducing kinase (NIK), respectively and indirect, which is interconnected with other cellular pathways, including MAPK, Rho, and phosphoinositide3-kinase (PI3-K) [24]. The NF-kB regulates more than 150 genes involved in anti-apoptosis, cell proliferation, inflammation and plays a key role in regulating the activation, survival, and differentiation of innate and adaptive immune cells. In RA pathogenesis, dysregulated NF-kB signaling contributes to activation of both immune and non-immune cells through transcriptional regulation of inflammatory mediators, including TNF-α, IL-1, -2, -6, -8, -9, -12, -18, -23, GM-CSF, VEGF, RANKL, MCP-1, MIP-2, CXCL1, CXCL10, RANTES, ICAM-1, VCAM-1, MMPs, and COX-2 [24]. On the other hand, inflammatory cytokines also modulate NF-kB through positive feedback, forming a vicious loop, which intensifies RA development [17]. At the same time, excessive NF-κB activation induces apoptosis of abnormal FLS cells in RA, which further accumulates in joint tissues and debris adheres to cartilage and bone, exacerbating the articular cartilage and bone destruction [17]. Along with that NF-κB dysregulation activates different types of T cells and also differentiate Th1 and Th17 cells by inducing IL-12 production and promotes IL-17 synthesis in Th17 cells, and thereby recruiting neutrophils and monocytes to sites of inflammation. Th17 cells contribute to inflammation by regulating expression of TNF, IL-1b, IL17, IL-21, and IL-22 [24]. The

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SYK (spleen tyrosine kinase) is a central molecule of B-cell receptor signaling and the level of phosphorylated SYK in peripheral blood B cells of RA patients has been dramatically increased. Strong positive autoantibodies against citrullinated peptides has been also established [230]. Relevant examples of plant derived molecules demonstrating their effect in *in vitro* RA models have been presented in Table 2.

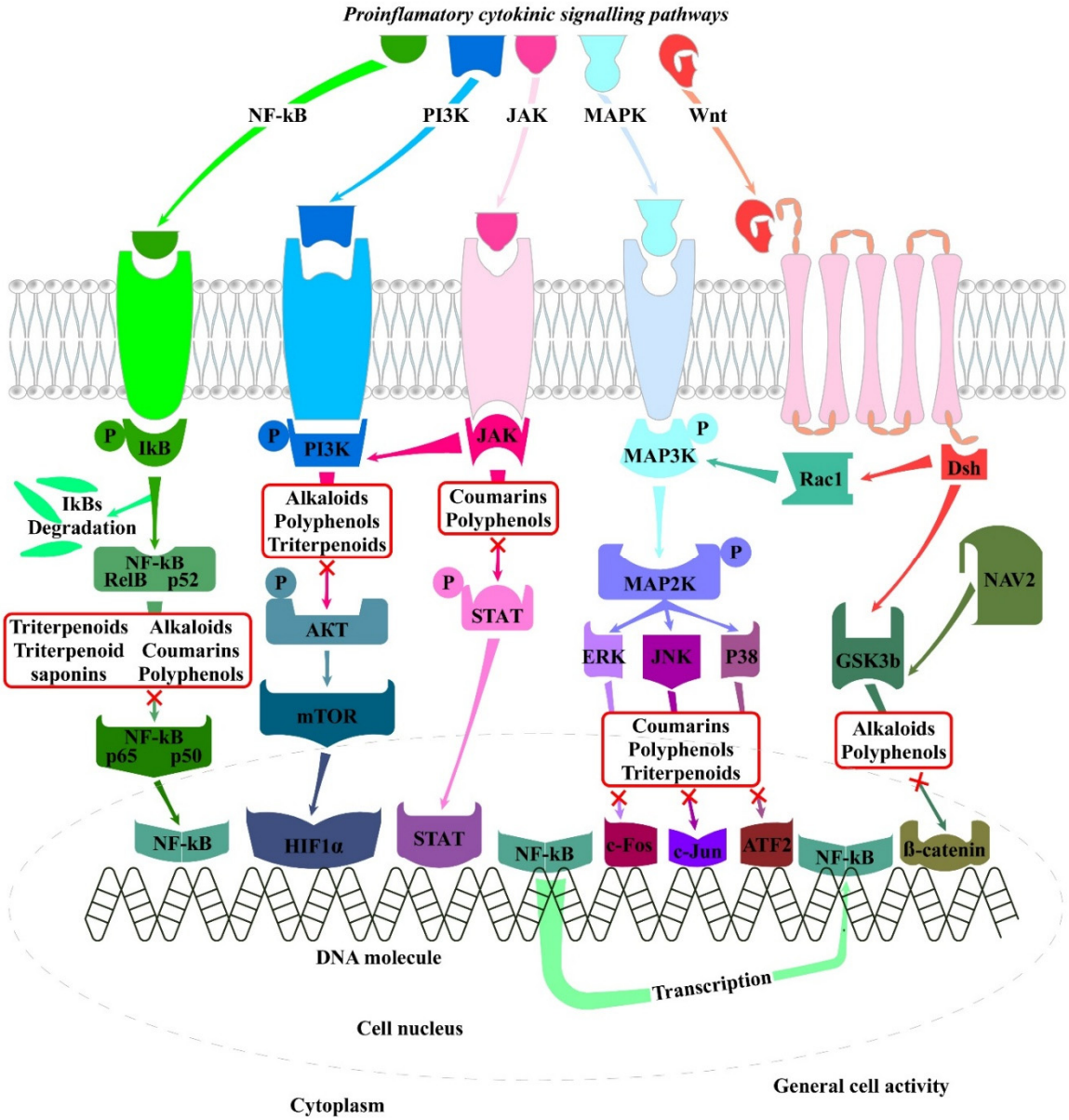

Figure 3. Signaling network in RA and its possible inhibition by plant-derived molecules. The red lines indicate the blocked signaling pathways.

The 70% ethanol extract of *Periploca forrestii* Schltr. rich of flavonoids revealed notable anti-RA activity. At concentrations from 25 to 500 ng/mL the extract mitigated the cell injury, reduced cell apoptosis and inflammation through reduced mRNA expression of *COX-2*, *iNOS*, *IL-6* and *IL-1β* in TNF-α stimulated L929, HEK293T and MH7A human RA-FLS by inhibiting the activation of NF-κB signaling [110]. The sesquiterpene lactones-enriched fraction from *Xanthium mongolicum* Kitag exhibited the strongest anti-RA, which dose dependently (from 1 to 640 μg/mL) decreased the expression of M1-related genes IL-6, -1β, TNF-α, -12b and iNOS through suppression of NF-kB signaling [111]. The ethanol extract of *Achyranthes aspera* L (doses from 100 to 300 μg/mL) revealed its potential as natural anti-inflammatory remedy in treatment of RA by downregulation of the mRNA expression levels of inflammatory genes, blocking the NF-kB promoter activity induced by TNF-α

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and inactivation of two upstream signaling molecules, such as Src and Syk kinases [246]. The mechanism of anti-RA activity of Siweixizangmaoru decoction (dose from 12.5 to 400 μg/mL) from Tibet revealed to include modulation of TNF-α, IL-1β, -6, -4, and -10, MMP-2, -3, -9, and MMP-13 through suppression of JAK2/STAT3 and NF-κB signaling [112]. The Chinese herb *Kadsura coccinea* (Lem.) A. C. Smith also revealed its anti-RA activity through inhibition of NF-κB and STAT1 signaling [113], while *Nyctanthes arbortristis* L from India reduced the production of various inflammatory mediators and factors *in vitro*, such as NO, ROS, iNOS, COX-2, TNF-α, IL-6 and -1β by inhibiting the activation of NF-kB [247]. Investigating the effect of *Geranium Wilfordii* Maxim. in MH7A cells was established that its anti-RA activity was due to modulation of the expression of Bax, Bcl-2, IL-6, -8, MMP-1, MMP-2, -3, -9, COX-2, and iNOS through inhibition of NF-kB and MAPK pathways [238]. The JAK/STAT pathway is another crucial signaling pathway deregulated in RA and governing cell differentiation, proliferation, with special emphasis of inflammation and immune functions [24].

The JAK family has four members, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), while the STAT family of TFs consists of seven members, namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Upon receptor ligation, JAKs are autophosphorylated, and recruit and phosphorylate members of the STAT family and many of the proinflammatory cytokines, such as TNF-α, IL-1β, -6, -7, -8, -12, -15, -17, -23, -32, IFN-γ and GM-CSF, that are highly expressed in RA are known to be regulated by JAK/STAT signaling pathway [17,24].

Tinospora cordifolia (Thunb.) Miers extract, rich of polyphenols effectively downregulated the level of pro-inflammatory mediators (IL-6, TNF-α, PGE2, COX-2, iNOs) and angiogenic factor VGEF through targeted the upstream kinases of the JAK/STAT pathway [248]. The acidified methanol extract of *Pennisetum glaucum* (L.) R.Br., rich of polyphenols demonstrated strong anti-RA activity *in vitro* by inhibiting MMP-9 and PTGS2 *via* suppression of JAK2 signaling pathway [249]. The water extract of *Pueraria montana* (Lour.) Merr. suppressed the inflammation, migration and invasion in human rheumatoid fibroblast-like synoviocyte line, MH7A through modulating the expression of Bcl-2, Cas-3, Cas-9, MMP-1, -9, IL-6, -8, -1β, TNF-α and SOCS1 [116]. In single osteoclasts cell culture, the classic Chinese herbal compound Yi Shen Juan Bi Pill relieved the symptoms of RA through regulating the bone immune microenvironment *via* JAK2/STAT3. *In vitro*, this compound decreased the number of $TRAP^+$ cells, the areas of bone resorption and inhibited the expression of RANK, NFATc1, c-fos, JAK2, and STAT3, while promoting the expression of IL-10 [250]. Similarly, another traditional Tibetan medicine (Shi-Wei-Ru-Xiang pills). In a co-culture system of IL-1β-stimulated synoviocytes or chondrocytes exhibited inhibition of chondrocytes apoptosis, associated with attenuation of inflammation by inhibition of the phosphorylation of p38, Erk1/2, and STAT3 [123]. Jolkinolide B (an ent-abietane-type diterpenoid found in *Euphorbia* plants) revealed its anti-RA potential at 1 μM concentration through suppression of TNF-α and IL-6 by decreasing the protein expression level of JAK2/STAT3 pathway *in vitro* [251].

Table 2. Plant-derived molecules targeting the major cytokines, TFs and signaling pathways in RA directly or indirectly *in vitro*.

Molecule	Dose, μM	Cell line	Targets	Main findings	Modulated pathway	Reference
Curcumin	50	MH7A	TNF-α, IL-6, IL-17	Inhibition of migration, invasion and inflammation	PI3K/AKT	[231]
Emodin	15	L929	IL-6, IL-1β, COX-2	Inhibition of inflammation	NF-κB	[232]
Ginsenoside compound K	30	Isolated FLS	FLUT1, HK2,	Inhibition of glycolysis	NF-κB	[233]

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			PKM1, PKM2			
Glytabastan B	3 and 6	SW982	TNF-α, IL-6, IL-8, COX-2 and MMP-1	Inhibition of inflammation and invasion	MAPK, PI3K/AKT, NF-κB	[234]
Isobavachalcone	20	MH7A	TNF-α, MAPK13, EGFR, PTGS2, MMP-3	Inhibition of migration, invasion and inflammation	PI3K/AKT, JAK/STAT	[235]
Kaempferol	10	HFLS-RA	IL-1β, MMP-2, -9, N-cadherin, vimentin	Inhibition of inflammation and abnormal proliferation	MAPK	[236]
Leocarpinolide B	20	SW982	IL-6, IL-8, IL-1β,	Inhibition of proliferation, migration, invasion and inflammation	NF-κB	[237]
Magnoflorine	10	MH7A	iNOS, COX-2, IL-6, IL-8, MMP-1, -2, -3, -9 and -13	Inhibition of proliferation, migration, invasion	PI3K/AKT, NF-κB, Nrf-2,	[238]
Nimbolide	1	HIG-82	MMP-2, IL-6, iNOS, COX-2	Reduction of inflammation	MAPK, NF-κB, Nrf-2	[239]
Quercetin	1.5	L929, HEK293T, MH7A	COX-2, iNOS, IL-6, IL-1β	Reduction of cell apoptosis, improvement of cell injury	NF-κB	[110]
Sappanone A	40	HFLS-RA	TNF-α, IL-1β, IL-6, IL-10, IL-17A	Inhibition of inflammation	JAK2/STAT3, PI3K/AKT, NF-κB	[240]
Shikonin	$1x10^{-7}$	MH7A	VEGF, VEGFR2, TNF-α, IL-	Inhibition of migration, invasion and adhesion	MAPK (ERK1/2, JNK, and p38)	[241]

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			1β, PDGF and TGF-β			
Scopoletin	30	HFLS-RA	IL-1β, TNF-α, MMP-3, MMP-9, COX-2, Bcl-2	Inhibition of proliferation, migration and invasion	NF-κB	[242]
Suberosin	5	RA-FLS	IL-6, IL-1β, TNF-α, IL-8, MMP-1, MMP-3, MMP-9, MMP-13	Inhibition of inflammation	JAK/STAT	[243]
Tectoridin	50	HFLS-RA	IL-1β, IL-6, COX-2, iNOS	Inhibition of inflammation	MAPK (ERK1/2, JNK, and p38)	[244]
Umbelliferone	20	HFLS-RA	IL-1β, TNF-α, MMP-3, MMP-9, COX-2, Bcl-2	Inhibition of proliferation, migration and invasion	NF-κB	[242]
Wilforine	0.4	Isolated FLS	IL-1β, IL-6, TNF-α, CCND1, GSK-3β, and c-Myc, MMP-3	Inhibition of inflammation and abnormal proliferation	Wnt11/β-catenin	[245]

The PI3K/AKT pathway regulates proliferation, metabolism, angiogenesis, and cell survival and is correlated with the occurrence and development of RA [17]. This pathway participate in the abnormal proliferation of FLS cells and synovial inflammation by stimulating the expression of inflammatory molecules like IL-1β, -6, -17, -21, -22, and TNF-α [17]. Abnormal PI3K/AKT pathway activation stimulate the expression of VEGF and HIF-1α to promoting angiogenesis, which not only disturbs the nutrition processes in the synovium, but also promotes glycolysis [27] and release diverse inflammatory mediators [17]. The PI3K/AKT activates mammalian target of rapamycin (mTOR) and further inhibiting autophagy in FLS, promoting continuous abnormal proliferation of synovial cells, and is also critical for the survival and differentiation of osteoclasts, aggravating RA [17].

Hedyotis diffusa Willd modulated its anti-inflammatory targets (TNF-α, IL-6, IL-17 and IL-10) *in vitro* through suppression of PI3K/AKT signaling pathway, thus revealing its anti-RA capacity at varying concentration between 0.5 and 2.0 mg/mL [17]. The ethanolic extract of *Ammopiptanthus nanus* (M. Pop.) Cheng f. inhibited the pathways PI3K/AKT/NF-κB closely related to RA by suppressing the

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expression of inflammatory cytokines IL-1β, COX-2 and iNOS *in vitro* used in concentration between 1 to 30 μg/mL [252].

The MAPK signaling pathway plays a key role in the pathological process of RA in terms of regulation of various cellular activities, including gene expression, metabolism, migration, survival, cell cycle progression, apoptosis, and differentiation and its over activation is closely correlated to the articular cartilage destruction and inflammatory hyperplasia of the synovial tissues. P38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) are the three main subfamilies of the MAPK pathway. The ERK1 and ERK2 are important for the regulation of cell differentiation, proliferation, and survival. On the other hand, the main effect of JNK MAPKs in RA is cartilage destruction mediated by MMPs. Similarly, P38 is linked to the inflammatory response in RA and activates many protein kinases and transcription factors that play key roles in the regulation of humoral and cellular autoimmune responses [17].

The dried roots of *Lithospermum erythrorhizon* Sieb containing shikonin as a major compound inhibited the migration, invasion and inflammation process related to RA *in vitro* through inhibition of angiogenic and inflammation mediators, such as VEGF, TNF-α and IL-1β [241]. The phenolic compound 5-hydroxyconiferaldehyde, isolated from the *Campanula takesimana* revealed its anti-RA potential by inhibiting the inflammatory response *in vitro* (inhibition of PGE2, iNOS, TNF-α, COX-2, IL-6, -1β) through suppression of several signaling pathways, such as MAPK, NF-κB, Nrf-2 [253]. The saponins rich fraction from Rhizoma Panacis Majoris exhibited its anti-RA potential through decreasing the expression of autophagy-related indicators (LC3II/LC3I, Beclin-1) and the corresponding signaling pathways, such as MAPK and PI3K/AKT [254].

The Wnt (Wingless/Integrated) signaling pathway takes part in a variety of pathological symptoms such as maintenance, differentiation, proliferation, and self-renewal in RA. The Wnt pathway also plays a key role in synovial inflammation and in the regulation of bone metabolism in RA [17]. A traditional Chinese Medicine Er Miao San (a mixture of Atractylodis Rhizoma and Phellodendri Cortex) revealed a promising anti-RA activity *in vitro* by decreasing angiogenesis and inflammatory microenvironment in MH7A cells by inhibiting the Wnt/β-catenin signaling [255]. The total saponins fraction of *Radix clematidis* have a pronounced anti-RA activity inhibiting the excessive proliferation of FLS during *in vitro* studies. At concentrations ranging from 0.5 to 1562.5 μg/mL inhibited c-Myc, cyclin D1, GSK-3β, and SFRP4 markers through Wnt7b/β-catenin [256].

The Notch signaling affects numerous processes of normal cell morphogenesis, including cell proliferation, the differentiation of pluripotent progenitors, apoptosis, and the formation of cell boundaries [17]. The Qi-Sai-Er-Sang-Dang-Song decoction (concentrations from 0.25 to 4%) inhibited the RA symptoms with focus on inflammation by inhibiting IL-6, -18 and -1β that were regulated by Notch/NF-κB axis [257]. In the range of 1 to 30 μM norisoboldine (an alkaloid compound isolated from Radix Linderae) inhibited synovial angiogenesis *in vitro* by decreasing VEGF expression and Notch1 signaling pathway [258].

5. In Vivo Studies Using Plant-Derived Natural Products for the Management of Rheumatoid Arthritis

Rheumatoid arthritis is a chronic autoimmune disorder characterized by various inflammatory processes causing pain, swelling, redness and malfunctioning of joints [259]. The ethiology of RA represents a complex combination of genetic and epigenetic predispositions as well as of environmental factors [260]. Despite the long list of medications for RA, most of them bring only partial relief to the patients and new therapeutic agents for treating the sources of disease and not just the symptoms are still needed [261,262].

The most frequently applied drugs for RA treatment are conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs), among which methotrexate (MTX) is the recommended first-line therapy for RA; biological DMARDs (bDMARDs) and targeted synthetic DMARDs (tsDMARDs) [263]. The treatment with csDMARDs ideally comprise MTX plus low-dose glucocorticoids. However, response to MTX varies and only after 6 months of therapy, 43% of the

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patients are classed as non-responders to MTX [264]. The bDMARDs include targeting monoclonal antibodies against TNF-α, IL-6, soluble receptor for TNF and T-cell co-stimulation. The tsDMARDs are inhibitors of the Janus tyrosine kinase family (JAK), which targets intracellular signalling of type I and II cytokines [263]. In addition to poor response, many patients often experience adverse events (AEs) such as gastrointestinal AEs and/or elevated liver enzymes [264], liver fibrosis, myelosuppression, pneumotitis [265] and are at a higher chance of developing infections, tuberculosis and certain malignancies, such as lung, breast, skin cancer and lymphoma [266].

Therefore, there is not only a clinical need to identify patients at high risk of non-response to DMARDs, but also to identify novel effective RA therapeutics without any side effects. In this regard, natural products with anti-inflammatory activity represent promising adjuvant agents or alternatives to RA therapeutics [4].

Experimental therapy with newly developed drugs in humans is limited for technical and ethical reasons. In this case, the rodent models are very useful because of low cost, homogeneity of the genetic background, and ease of handling. Although the animal models mimic a part of a disease and never duplicates or reproduces the entire human pathology, they are very useful to test the hypothesis of the global effect of a molecule.

The animal models of RA can be classified into two broad categories of: (A) induced animal models for RA, (B) genetic models of RA. The induced models vary according to the used chemical agent with arthritogenic properties for instance, collagen, pristane, complete Freund's adjuvant (CFA), Cartilage oligomeric matrix protein (COMP). In the case of the genetic models, the animals are either deficient in (knockout) or transgenic for a specific gene of interest [267]. The genetic models have a valuable role in the arthritis research. The information regarding the role of depleted or introduced particular gene, gives a valuable insight to the process of inflammation and regulation. These models serve as a tool to study the effect of therapeutics in mice prone to developing joint inflammation spontaneously and to understand varying manifestations of autoimmunity in general. In the present report, we summarized the widely used rodent models of RA for testing the therapeutic potential of the biologically-active compounds.

5.1. Collagen Induced Arthritis Model

Known as a gold standard of the *in vivo* models, the collagen induced arthritis (CIA) model sharing many similarities with the pathological and immunological features of the human RA. The clinical signs of this polyarthritis model are inflammation of the synovium, cartilage destruction and bone erosion [268]. The antibodies play an important role in the inflammatory phase of CIA. The immunization with heterologous type II collagen in complete Freund's adjuvant (CFA) to genetically susceptible mice strains with MHC haplotypes H-2^q or H-2^r (DBA/1, B10.Q, and B10.RIII) leads to generation of autoantibodies against self-antigens and collagen [153]. It was also shown that C57BL/6 (B6; H-2^b) [269] can also develop CIA with a high incidence (60–70%) and sustained severity dependently on B and CD4 + T cells. The susceptibility to CIA requires more than MHC class II haplotypes restriction but also TCR, and non-MHC susceptibility genes. This is also confirmed by the fact that the passive transfer of collagen type II specific T cells doesn`t induce severe inflammation compare to the transfer of collagen type II specific antibodies. The dominant pathological role of Th1 and Th17 cells is well known, but the antibodies against collagen type II seems to have primary role in the immunopathogenesis of this model. CIA is an important rodent model for the analysis of non-MHC genes and their role in RA development [153].

5.2. Collagen Antibody Induced Arthritis Model

The passive transfer of commercially available monoclonal antibody cocktail to collagen type II presents an alternative to the CIA, significantly reducing the study duration and cost while increasing disease induction and synchronicity among individual animals. This model can be induced in MHC haplotypes non- susceptible to CIA. Terato et al. [270] have shown that by *i.v.* injection of a combination of 4 different monoclonal antibodies can be induced antibody-mediated CIA. Further

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research by Nutty Nandakumar and Rikard Holmdahl [271] has improved the antibody cocktail by selecting epitope specific antibodies, which is critical for their pathogenicity. The combination of monoclonal antibody cocktail and LPS leads to the development of severe and persistent arthritis to nearly 100% of the mice. As advantages of the CAIA model can be pointed out: length of the development (typically within days instead of weeks as in the classic CIA model); synchronization between the animals into the group (the onset of the disease is 2 days after LPS injection); Susceptibility (not only CIA-susceptible mice strains, but also some CIA-resistant mice, such as Balb/c and C57BL/6) [272]. The pathology of this model and the induction of the inflammation is expressed in the formation of immune complexes with the collagen type II on the cartilage or synovium, and activation of the classical and alternative complement` pathways. Additionally, the antibody complexes may also activate the monocytes in the joint via Fc receptors, and the releasing of pro-inflammatory cytokines (i.e. TNF-a and IL-1) recruit neutrophils and macrophages [273]. This model of RA can be used to studying inflammatory mechanisms in arthritis and to screen candidate therapeutic agents.

5.3. Adjuvant Induced Arthritis Model

The adjuvant induced arthritis model (AIA) present an efficient way to enhance both the cell-mediated and humoral immune responses towards the antigens by emulsified them in Complete Freund's adjuvant (CFA) [274]. AIA is severe, sub-chronic arthritis, characterized by persistent inflammation with numerous systemic alterations as synovial hyperplasia, cartilage and bone damage, as well as edema and deformation. The massive leucocyte infiltration leads to increased chemokine and cytokine levels (as IL-1 and TNF-α), and release of reactive oxygen species (ROS) [275]. AIA is suitable as a model for screening and testing anti-arthritic drugs. Its manifestations are very similar to those in the human RA [276].

5.4. Pristane Induced Arthritis Model

Pristane induced arthritis (PIA) is severe and chronic inflammatory model. The introduction of Pristane, a synthetic mineral oil (2,6,10,14-tetramethylpentadecane), induce an acute arthritic, massive formation of osteoclasts, bone erosion and new bone formation, and also an inflammatory cell infiltration. It`s characterized with prolonged and delayed clinical features (from weeks to months). Serological parameters as rheumatoid factor (RF), antibodies against heat shock proteins and collagen type I and II, and the elevated levels of cartilage oligomeric matrix protein can be analyzed. The development of PIA is joint-specifically regulated by T cells and by MHC genes and fulfill the clinical criteria for RA. This model is useful for validation of new drug candidates [276–279].

In the recent years, different herbal extracts have been reported to have beneficial effect on RA development in animal models of the disease. The rhizome of *Acorus gramineus* Sol. ex Aiton (a widely used plant in traditional Chinese medicine) has shown therapeutic effect in CIA mouse model of RA – it decreases IL-6 and TNF-α and ameliorates swelling of the hind limb [280]. The fruit peel extract of *Annona squamosa* L contains various immunomodulating chemical substances that have anti-RA functions - decreased leucocyte levels in the serum and decreased necrosis in the paws are observed after its administration in CFA-injected mice [281]. Another report pointed to the beneficial effect of *Saururus chinensis* (Lour.) Baill leaves extract on type II collagen-induced arthritis in mice [282]. Authors concluded that *S. chinensis* extract administration has positive effect on different RA manifestations – increased serum levels of IL-6 and TNF-alpha, swelling of hind limbs and accumulation of inflammatory cells in the synovial membrane. In their research, Kim et al. investigated the therapeutic potential in RA of extract mixture of two folk remedies - *Cudrania tricuspidata* (Carrière) Bureau and *Stewartia koreana* Maxim. The results showed that treatment with the extract mixture has strong anti-inflammatory effect as it reduced nitric oxide levels, tumor necrosis factor, IL-6 and IL-1 levels [283]. Another extract, rich in antioxidants, catechins and proanthocyanidins is the grape seed proanthocyanidin extract. This extract expresses therapeutic

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effect in CIA mouse model of RA by regulating the TLR4-MyD88-NF-κB signaling pathway which further resulted in improvement of clinical features and joint histology [284]. Thorn extract of *Gleditsia sinensis* in combination with *Lactobacillus casei* has anti-inflammatory effects in mouse model of type II collagen-induced RA [285]. The amelioration was observed in decrease in serum nitrite and total cholesterol as well as in pro-inflammatory cytokine (TNF-αand IL 6) levels. Another interesting observation is the report of Allam et al. concerning the therapeutic effect of the phenolic substance ellagic acid in CFA-injected mice [286]. Authors clearly demonstrated that treatment with ellagic acid attenuated paw swelling and bone dysfunction by modulating pro- and anti-inflammatory cytokines.

In addition, the beneficial effect of different plants has been reported and in rat models of RA. Wood bark extract from the fruit Durian (*Durio zibethinus* Murr.) has shown therapeutic effect in CFA injected-rat rats - improvement of hind limb swelling and of histopathological changes and suppression of iNOS expression due to the presence of various phenols, alkaloids, tannins, terpenes, saponins, and flavonoids in the extract [287]. Root and leaf extracts of *Chloranthus serratus* (Thunb.) Roem. & Schult. (a traditional Chinese medicine plant) also showed beneficial effect in RA treatment. The administration of extract in CFA-injected rats resulted in inhibition of secretion of pro-inflammatory cytokines [288]. Finally, ethyl-acetate extract from the root of *Caragana sinica* (Buc'hoz) Rehder (herbal product in traditional Chinese medicine) has been reported to have therapeutic efficacy for RA treatment in adjuvant-induced arthritis in rats. The administration of extract resulted in reduced paw swelling and protected bone structures due to negative regulation of the NF-κB pathway [289].

All these results pointed herbal extracts as promising therapeutic agents in experimental mouse and rat models of RA with unexploited potential for further clinical implications (Table 3).

Table 3. Mode of action of different herbal extracts reported to have beneficial effect on RA in animal models.

Host	Biological agent	Mode of action	Reference
Mouse	*Acori Graminei*	Reduction of inflammation indicators including IL-6 and TNF-*α*	96
Mouse	Fruit peel extracts of *Annona Squamosa L.*	Decrease in the leukocytes in the serum	97
Mouse	*Saururus chinensis*	Reduction of inflammatory cytokines	98
Mouse	*Cudrania tricuspidata* and *Stewartia koreana*	Decrease in inflammatory cytokine levels, NOS inhibitors	99
Mouse	Grape seed proanthocyanidin extract	Inhibition of TLR4/ MyD88/NF-κB signaling pathway.	100
Mouse	*Gleditsia sinensis* thorn extract fermented by *Lactobacillus*	Reduction of inflammatory cytokine levels	101
Mouse	Ellagic acid	Downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines	102
Rat	Duran wood bark extract	iNOS suppression/NOS inhibitor	103
Rat	*Chloranthus serratus*	Inhibition of the releases of inflammatory cytokines and amelioration of antioxidant capacity	104

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Rat	*Caragana sinica*	Negative regulation of the NF-κB pathway	105

6. Conclusions and Future Perspectives

Rheumatoid arthritis is a chronic autoimmune disorder characterized by inflammation of the joints, resulting in pain, joint swelling and finally joint damage and bone erosion. Profound insight into RA etiology reveals that many genetic, epigenetic changes, posttranslational modifications and environmental factors, including complex interactions at mucosal levels between microbiome and host immune cells provoke the pathogenesis and progression of RA. In spite of the numerous drugs and novel biological therapies, they have several limitations and drawbacks, including disease recurrence and adverse effects due to long-term use. Rheumatoid arthritis has turned from a highly disabling disease for which no effective remedies existed to a disorder that can be controlled well, with many patients reaching remission. However, still many unmet needs remain, since not all patients reach sustained clinical remission and even about 25% still suffer from moderate or even high disease activity; even some patients still do not reach low disease activity; great similarity of response rates to the various targeted therapies has been frequently observed. Therefore, it is still needed to uncover the cause(s) for RA in order to assure cure or at least prevention; novel therapies need to be established; different and yet unidentified signaling pathways in non-responders need to be identified.

Currently, although still underestimated plant-based natural extracts and molecules, including flavonoids, terpenoids, alkaloids and many others possessing anti-inflammatory, immunomodulatory, anti-oxidant and anti-apoptotic activity have demonstrated excellent curative effect on autoimmune diseases, including RA and even some of them have already been used in treating RA patients. Major challenges using medicinal plants are their complexity related to identification of the bioactive components, understanding the mechanisms of the phytopharmaceutical activity and their poor availability. The inclusion of plant-derived extracts and molecules into novel delivery systems, such as nanoparticles improves their pharmacological and therapeutic properties. Nanoencapsulation seems to be a promising strategy to enhance the permeability, solubility of the molecules protecting them from degradation and improving their bioavailability. Therefore, this encapsulation allows precise dosage, prolonged and controlled release of the phytochemicals, as well as, reducing dosage regimen and drug toxicity, and improving patient compliance. The use of nanocariers offer an innovative platform for the topical delivery of plant-derived molecules in the treatment of RA. Using different "omics" technologies, such metabolomics, as well as, 2- and 3-D *in vitro* models and different panels of *in vivo* models is also a possibility to overcome some of the challenges associated using plant-derived products and finally decrease the failure of potential new therapies in clinical trials. Combining different 3D tissue models with microfluidic devices could be the next generation *in vitro* approach to study the complex cross talk between tissues/organs and immune system, including the spreading of (auto)immune reactions across different organs. Although, the broad diversity of conventional RA mice models, they are partially suited to preclinical testing of cell-based therapies. For example, spontaneous mice models are suitable to study autoimmunity, while induced models are more focused on the effector phases of immune-mediated arthritis. For that reason, the translational research in humanized mice models that accurately mirrors autoimmune processes of human RA and permits its modulation by the transfer of human immunoregulatory cells are expected to be a powerful tool for preclinical evaluation of cell-based immunotherapies. Human-based approach will provide opportunities to identify objective patient-related biomarkers to elucidate disease subtypes and treatment response, but will also enable strategies for the management of patients who are 'refractory' or resistant to available treatments.

As future outlook, plant-derived products also contain prebiotic components, whose interaction with the host microbiome might have a significant impact on health and disease. A future task for researchers in the field will be to identify how these parameters interact to trigger an autoimmune

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inflammatory response and the development of RA and would further help to optimize the selection of plant-derived products for therapy and define their mechanisms of activity. In the near future additional research efforts might be invested in combining phytotherapy with stem-cell research, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) genome editing or gene therapy that would provide a long-term therapeutic advantage for RA patients following evaluation of safety, ethical, and medical concerns.

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Abbreviations

ACPAs	Anti-citrullinated protein antibodies
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
Anti-CarP	Anti-carbamylated protein antibodies
CCR6	Chemokine receptor 6
cDMARDs	Conventional disease modifying anti-rheumatic drugs
COX-2	Cyclooxygenase-2
CRP	C-creative protein
CTLA-4	T-lymphocyte associated protein 4
CXCL12	Chemokine C-X-C motif ligand 12
DALYs	Disability-adjusted life-years
DNMT	DNA methyltransferases
ECM	Extracellular matrix
ESR	Erythrocyte sedimentation rate
FGFs	Fibroblast growth factors
FLS	Fibroblast-like synoviocytes
GC	Glucocorticoids
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HB-EGF	Heparin-binding EGF-like growth factor
HDAC	Histone deacetylases
HLA-DR	Human leukocyte antigen D-related
IgG	Immunoglobulin G

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IL	Interleukin
IRF-5	Interferon regulatory factor 5
JAK/STAT	Janus-activated kinase signal transduction and activator of transcription
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MDC	Myeloid dendritic cells
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
MTX	Methotrexate
NF-κB	Nuclear factor kappa B
NF-κβ	Nuclear factor kappa-B
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OPG	Osteoprotegerin
PADI4	Peptidyl arginine deiminase, type IV enzyme
PBMCs	Peripheral blood mononuclear cells
PDC	Plasmacytoid dendritic cells
PI3/AKT	Phosphatidylinositol 3 kinase-AKT
PlGF	Placenta growth factor
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor-B ligand
RF	Rheumatoid factor
ROS	Reactive oxygen species
SAM	S-adenosine methionine
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SYK/BTK	Spleen tyrosine kinase)/Bruton's tyrosine kinase
TBX5	T-box transcription factor 5
Tfh	T follicular helper
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor alpha
TRAF1	Tumors necrosis factor-receptor associated factor 1
Treg	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
Wnt/β-catenin	Wingless/Integrated

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