The New and Evolving Science of IL-6 in Rheumatoid Arthritis

Neutrophils in RA and the Role of IL-6

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SANOFI GENZYME REGENERON

Dear Colleagues,

This is a very exciting time in the field of rheumatoid arthritis (RA). The more we understand from basic and clinical research about the pathogenesis of RA, the more equipped we are to understand this disease. We now know that cytokines play many key roles in the inflammation that drives RA. One such example is interleukin-6 (IL-6), a multifunctional cytokine that contributes to chronic inflammation in patients with RA.

Regeneron Pharmaceuticals and Sanofi Genzyme are excited to bring you additional educational material describing some of the fundamental immunology as well as clinical pathology we see in our RA patients through a series of scientific monographs entitled *The New and Evolving Science of IL-6 in Rheumatoid Arthritis*. In the first installment, we reviewed the signaling mechanisms of IL-6 that allow it to have widespread effects in RA. In the second installment, we reviewed the contributions of the IL-6 pathway to bone resorption in RA. In the third installment, we reviewed how persistently elevated IL-6 signaling may contribute to both articular and systemic manifestations of RA. In the fourth installment, we reviewed the roles of IL-6 in both innate and adaptive immunity in RA. In this installment, we discuss the effects of IL-6 on neutrophils in RA.

We hope you find this latest installment informative and engaging.

Sincerely,

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Dr Calabrese and Dr Choy were provided honoraria by Sanofi Genzyme and Regeneron Pharmaceuticals.

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Introduction

Rheumatoid arthritis (RA) is characterized by a persistent activation of innate and adaptive immune responses, leading to autoimmunity, chronic inflammation, joint destruction, and systemic manifestations such as bone loss and induction of the acute phase response.[1] Activation of the immune system in RA is largely driven by a disruption in the balance between the pro- and anti-inflammatory cytokines that comprise the complex signaling network between immune cells.[2] For example, cytokines such as interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-1, and IL 17 are known to have central functions as proinflammatory effectors and are often found in abundance in patients with RA.[3-6] A relative upregulation in the expression of these proinflammatory mediators, together with a relative downregulation of anti-inflammatory mediators such as IL-10 and IL-11, can upset the cytokine balance and generate a positive feedback loop that perpetuates inflammatory conditions.[1,2] While genetic and environmental factors can contribute to the disruption of cytokine balance, there is no single known trigger for RA induction.[2] Instead, as is characteristic of autoimmune disorders, initiation of disease may be due to a loss of self-tolerance over time, which perpetuates the imbalance of cytokines and contributes to a cycle of progressive, and eventually chronic, inflammatory conditions.[1]

One proinflammatory cytokine that is of particular interest in the pathogenesis of RA is IL-6, which interacts with virtually all cells of the innate and adaptive arms of the immune system via its versatile signaling mechanism, which has been reviewed in a previous monograph titled *The Roles for IL-6 in Both Innate and Adaptive Immunity in RA*.[7,8] In RA, persistently elevated IL-6 signaling significantly contributes to chronic inflammation by triggering processes within, and perpetuating interactions between, the innate and adaptive systems.[7,9] Among the innate cells that are highly influenced by IL-6 are neutrophils—the first cells to arrive at sites of infection and inflammation.[7,10] Neutrophils have long been recognized as principal components of host defense; indeed, pathogen recognition and destruction are among their essential functions.[11] An increasing body of evidence that points to the complexity of neutrophils, however, has led to an advanced understanding of their roles beyond protection against pathogens.[12] It is now acknowledged that the neutrophil population may consist of several subsets with various functions and differential activation based on stimulus and microenvironment, and these cells might have diverse roles including (but not limited to) immunosurveillance, regulation of adaptive immunity, wound healing, initiation and maintenance of inflammation, and immune tolerance.[4,13,14] The role of neutrophils in these processes has become a topic of great interest, mainly because "breaking tolerance" to autoantigens represents a defining event in the development of autoimmune conditions such as RA, and the toxic nature of neutrophils' cellular contents can contribute substantially

to tissue damage and the development of chronic inflammation.[4,10,15,16] Neutrophils, and the role of IL-6 in driving their pathogenic behavior in RA, will be described in more detail herein.

Neutrophils in RA Pathology: Autoantibodies

A hallmark of autoimmunity is the generation of autoantibodies. In RA, autoreactive immunoglobulin (Ig)M and IgG rheumatoid factor (RF) immune complexes, as well as anticitrullinated protein antibodies (ACPAs), are detected in the majority of patients with RA.[2,10] ACPAs, which are present in approximately 70% of the RA population, are considered a marker of aggressive and erosive disease, and are often detected before the onset of symptoms, suggesting a role in driving disease progression.[17-20] ACPAs preferentially target epitopes patterned with post-translationally modified (citrullinated) residues—ie, hypercitrullinated proteins—many of which have been identified. However, their origin and specific role in driving the pathogenic ACPA response remain to be fully elucidated. It has been proposed that neutrophils are a likely source of these hypercitrullinated neoantigens, which may be exuded into the extracellular space as part of unique structures known as neutrophil extracellular traps (NETs).[10]

Autoimmune NETs as a Source of Autoantigens

NETs may be released during a process known as NETosis, a form of cell death distinct from apoptosis and necrosis in which the granular and cytosolic contents of the cell are emitted into the extracellular space, together with decondensed chromatin and other nuclear material **(Figure 1)**.[10] NETs were originally described as a strategy employed by neutrophils to immobilize and kill invading pathogens; recent studies have revealed that a broader range of stimuli—including reactive oxygen species (ROS), antibodies and immune complexes, and certain genetic or environmental factors—may induce NET formation.[21-26] Evidence obtained from investigation suggests that the contents of a particular NET may depend on the specific stimulus. For example, a notable difference between pathogen-induced NETs and autoimmune-associated NETs is the presence of hypercitrullinated proteins in the latter, but not in the former **(Figure 1)**.[27] These and other observations have contributed to a rapidly evolving field of investigation into the precise mechanisms of NET release and neutrophilic cell death; the field would welcome a new lexicon that makes distinctions between the different forms of NETotic process. In this monograph, we use the term "autoimmune NETs" to describe those triggered by RA-associated stimuli. Research in this field is progressing swiftly, and important studies conducted to date have suggested

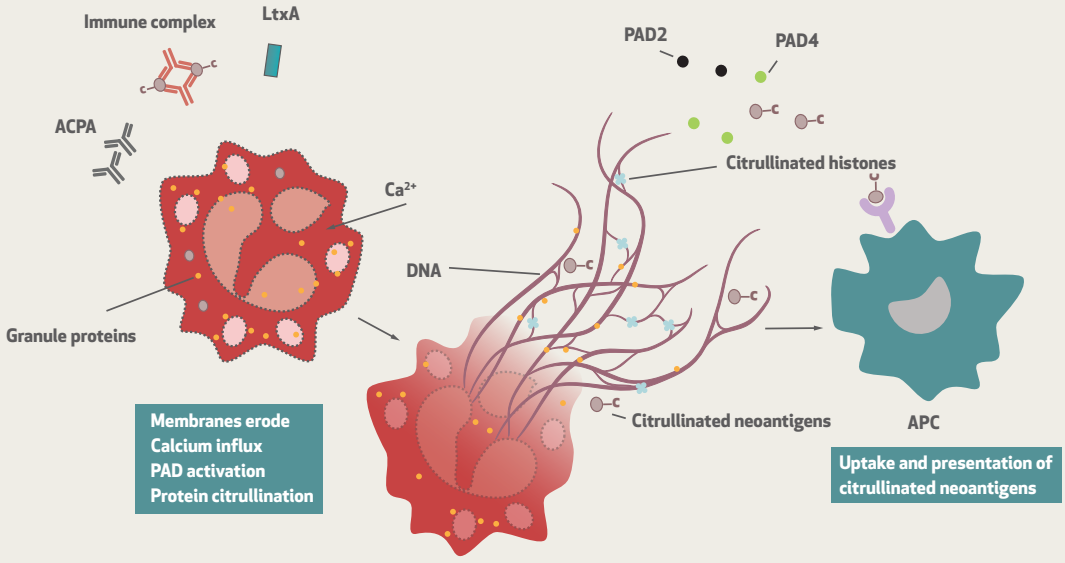

Figure 1. A Model for Autoimmune NET Release.[10,24,38,39] Autoimmune NET formation may be initiated by external stimuli such as immune complexes, ACPA, or bacterial toxins (eg, LtxA). Calcium influx leads to activation of PAD enzymes, which catalyze deimination of arginine residues on histones and other intracellular proteins. As a result of the loss of histone positive charge, chromatin decondenses, and erosion of the intracellular membranes allows mixing of the granular contents with DNA, other nuclear material, and citrullinated neoantigens. These contents, along with activated PAD_2 and PAD_4, are released when the outer membrane ruptures, either affixed to the chromatin scaffolding (as NETs) or as diffuse molecules, providing a source of autoantigens for uptake and presentation by APCs.

that certain factors that contribute to the development of RA could be related to their ability to specifically stimulate the formation of autoimmune NETs.[28]

In RA, several lines of evidence support the role of neutrophils and NETs in disease pathogenesis. Neutrophils are the most abundant cells in RA synovial fluid, and when they are isolated from either the synovial fluid or the peripheral blood of patients with RA, neutrophils have been shown to undergo a form of NETosis, even in the absence of external stimuli.[10,24] The percentage of NET-forming (NETting) neutrophils was positively correlated with high ACPA titers, potentially indicating a more aggressive disease phenotype. Furthermore, in co-culture experiments, NETs derived from RA synovial neutrophils were able to activate RA fibroblast-like synoviocytes (FLS), significantly upregulating mRNA and protein synthesis of key proinflammatory molecules, including IL-6 and IL-8.[24] Given the established role of FLS in mediating tissue damage in RA, these observations suggest a pathogenic role for NETs within the RA joint.

In addition to mediating local effects, NET-forming neutrophils may contribute to some of the systemic manifestations observed in RA. For instance, the degree of NETosis

in the peripheral blood of patients with RA was shown to correlate with markers of systemic inflammation, including increased levels of serum C-reactive protein (CRP) and IL-17, and elevated erythrocyte sedimentation rate (ESR).[24] NETs were also detected in rheumatoid nodules and in the skin of patients with comorbid neutrophilic dermatoses, a cutaneous manifestation of RA.[24,29] Several links between NETs and thrombosis have been identified: NETs can serve as a structural scaffold for thrombus formation, and chromatin and histones are known to initiate coagulation.[30-32] In addition, the NET-associated proteins neutrophil elastase and cathepsin G promote clot formation through degradation of endogenous anticoagulants.[33] Finally, NETs can express high levels of tissue factor, which activates the coagulation cascade locally.[31] Given the potential contributions of neutrophils and NETs to coagulation mechanisms, it is conceivable that excessive neutrophil activation and heightened NET formation could be related to an increased risk of cardiovascular (CV) complications.[34-36] These observations support the broad role of neutrophils in propagating not only local, articular responses, but also in systemically regulating the various components of inflammatory and immune responses, and in influencing such diverse processes as hematopoiesis, angiogenesis, and fibrogenesis.[37]

Generation of Hypercitrullinated Neoantigens

Autoimmune NET formation appears to be initiated by histone citrullination, which can be catalyzed by PAD4, an isoform of the peptidyl arginine deiminase (PAD) family of enzymes whose activity is in part regulated by calcium flux. PAD4 and other PAD isoforms also citrullinate other proteins, some of which are known targets for ACPA.[39] Given the early association between ACPA and RA, several intriguing PAD-related theories have emerged that may help to explain the connections between certain genetic, environmental, and pathogenic factors and the development of RA. For instance, the presence of a C1858T single nucleotide polymorphism (SNP) in *PTPN22*, a gene encoding a tyrosine phosphatase that inhibits PAD4 activity, carries a high risk for RA development, with an odds ratio of 1.5–2. When the genetic susceptibility allele human leukocyte antigen (HLA)-DRB1 shared epitope (SE) is also present, the C1858T SNP increases the risk of RA by 20–30-fold, and this synergy is primarily restricted to the ACPA-positive RA population. It was suggested that the C1858T SNP increases the risk of RA by impairing the ability of PTPN22 to physically bind and inhibit the activity of PAD4, resulting in enhanced protein citrullination and NET formation.[40] In a separate study, an RA-prone haplotype of the *PADI4* gene was found to stabilize neutrophilic PAD4 transcripts and was associated with high ACPA titers in RA serum samples.[41] Another group found that

$PAD3$/$PAD4$ cross-reactive antibodies were able to increase the efficiency of $PAD4$ by enhancing its calcium sensitivity. The authors speculated that these data indicate a feed-forward loop of citrullinated autoantigen generation that could help drive disease progression, and indeed, RA patients with these autoantibodies had an increased propensity for radiographic damage.[42]

The most widely recognized environmental risk factor for RA is smoking, and patients who smoke have higher levels of activated PAD2 and citrullinated proteins in bronchoalveolar lavage cells compared with nonsmokers.[1,43] Although it has been demonstrated that nicotine can bind neutrophil acetylcholine receptors to induce a NETosis-like pathway, evidence defining the impact of smoking on neutrophils in RA is limited.[44] A more direct link between neutrophil-associated hypercitrullinated neoantigens and RA development has been hypothesized in the context of periodontitis, which has emerged as a strong risk factor for RA. Exposure to *Aggregatibacter actinomycetemcomitans* (*Aa*), a Gram-negative facultative bacterium frequently found in the oral microbiome in patients with periodontitis, was found to correlate with ACPA and RF levels, particularly in the presence of the susceptibility allele HLA-DRB1-SE. Neutrophils exposed to *Aa* extruded NETs with patterns of PAD- and calcium-dependent protein hypercitrullination that were analogous to those of NETs isolated from RA synovial fluid. The *Aa*-induced NET-forming mechanism was shown to involve the pore-forming virulence factor Leukotoxin A (LtxA), which binds the β2 integrin receptor on neutrophils to induce membrane permeabilization, calcium influx, and $PAD4$ activation.[38] These data further support the importance of neutrophils—as well as the microbiome—in the pathogenesis of RA, and implicate NETs as a potential source of neoantigens that drive ACPA generation.

NETs and ACPA Generation

Patients who carry certain susceptibility alleles within the HLA-DR shared epitope are strongly predisposed to developing ACPA-positive RA.[45] This association may be related to an increased affinity of the HLA $P4$ binding pocket for citrullinated peptides, which could lead to high-density presentation of the HLA-peptide complex on the surface of antigen-presenting cells (APCs), enabling T-cell activation.[45,46] However, while the prevailing theories for the association between ACPA and HLA-based genetic disposition is based upon the antigen-presenting capabilities of HLA molecules, definitive evidence of the exact mechanisms leading to ACPA generation remains elusive.[47,48] It is known that, in RA, ACPAs target the citrullinated forms of certain proteins, including vimentin, antithrombin, fibrinogen, and α-enolase, which have been found immobilized within NETs.[24,39,49] Importantly, some of these citrullinated proteins, as well as the activated forms of PAD2 and $PAD4$, can also be released during NETosis as diffuse molecules **(Figure 1)**.[39] These soluble PAD enzymes could further contribute to the

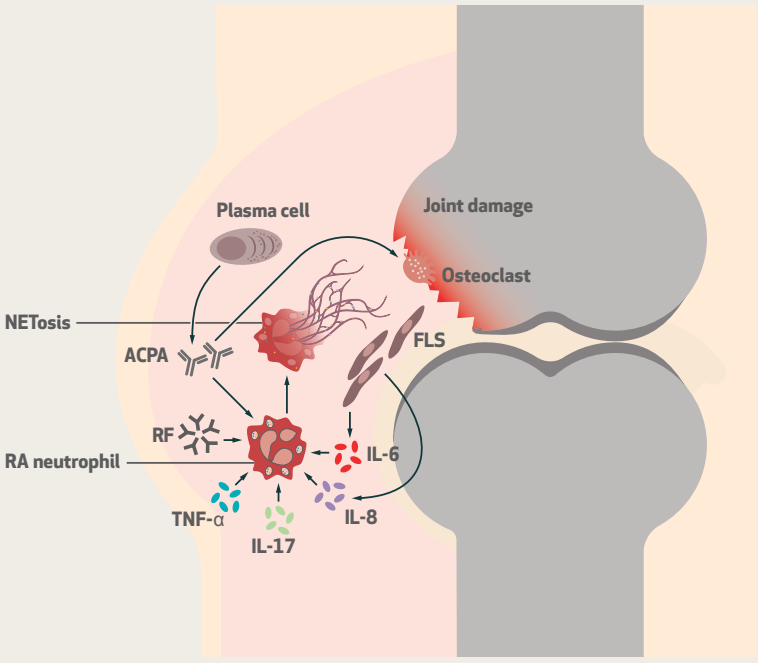

Figure 2. Feed-forward mechanisms of neoantigen formation, autoreactivity, and inflammation in RA.[8,24,38,50,60] Proinflammatory cytokines, immune complexes, ACPA, and other factors can influence NET release in RA. Autoimmune NETs may provide a source of autoantigens that can trigger ACPA production by plasma cells, which may further induce NET release and contribute to osteoclast formation and bone resorption. NETs also stimulate the secretion of IL-6 and other cytokines by FLS, which can facilitate tissue damage and further induce NET formation.

generation of autoantigens by citrullinating proteins that are primarily found in the extracellular synovial space (eg, fibrinogen and collagen).[39]

Interestingly, ACPAs may indirectly promote their own generation by inducing NET formation. IgG and IgM fractions isolated from the synovial fluid and peripheral blood of patients with RA, but not from healthy controls, dramatically induced NETosis in cultured neutrophils; antibodies against citrullinated vimentin also induced NET formation.[24] Thus, a perpetuating pathogenic mechanism is possible in RA, where neutrophils are central in driving the cycle of neoantigen formation, autoreactivity, and inflammation leading to joint destruction **(Figure 2)**.

IL-6-Mediated Regulation of NET Formation

Due to the inherent challenges of pursuing investigation into human neutrophils, definitive support for the direct regulation of NETs is limited. However, substantial evidence directly or indirectly obtained implicates IL-6 in multiple neutrophil processes including differentiation, migration, activation, apoptosis, and, more recently, NET emission.[6,7,50] Specifically, in neutrophils isolated from healthy subjects, autocrine IL-6 signaling induced NET formation to a level comparable to that induced by bacteria-associated lipopolysaccharides (LPS).[50] One study found that dampening IL-6 signals reduced the tendency of RA neutrophils to exude

NETs, possibly due to the key role of IL-6 in the expression of the NET-inducing molecules neutrophil elastase and myeloperoxidase (MPO).[51] IL-6 may also be indirectly involved in the regulation of intracellular calcium levels, which influence PAD activity: healthy neutrophils stimulated with IL-6 in vitro produced elevated levels of platelet-activating factor (PAF), which correlated with an increase in cytosolic calcium.[52]

An underlying aspect of the pathology of RA is the induction of feedback loops that can perpetuate or amplify inflammatory responses.[1] NETs may play a part in these loops due to their ability to influence FLS activation and, potentially, T- and B-cell activation; IL-6 may cooperate with NETs through its actions on similar cell types. For instance, IL-6 induces B cell maturation into plasma cells that can secrete ACPA, which may then induce NET release.[6,8,24,53] NETs were found to stimulate IL-6 production in RA-derived FLS in one study; however, in another study, IL-6 induced NET formation in healthy neutrophils in vitro.[24,50] Thus, IL-6 may act as a messenger between neutrophils and other effectors of RA pathogenesis and may facilitate destructive feedback loops that lead to disease progression **(Figure 2)**.

Regulation of Neutrophil Trafficking and Cytotoxicity in RA

Neutrophils are produced in the bone marrow and, under normal conditions, a steady concentration of cells is maintained in the circulation and in "marginated pools," which are reservoirs of neutrophils travelling slowly through the bone marrow, liver, spleen, and lungs.[14,54] Circulating neutrophils are generally quiescent, but can become rapidly activated in response to signals received from the endothelial lining of the vessel wall, or by encountering pathogens.[55] These signals can trigger extravasation, an egress of neutrophils from the vasculature into the surrounding tissue.[13] A hallmark of RA is an increase in the number of circulating neutrophils, accompanied by an influx of neutrophils from the circulation into the synovium, where they mediate localized inflammation and tissue damage.[10]

Neutrophil Concentration and Localization

Neutrophils have a relatively short half-life—remaining in circulation for 24 hours before undergoing apoptosis, and therefore continuous generation of neutrophils in the bone marrow is necessary to maintain a steady state.[10,14]

IL-6 has been shown to increase neutrophil efflux from the bone marrow, and can also cause a rapid shift in neutrophil localization from the marginated to the circulating pools.[56,57] Thus, the markedly upregulated IL-6 levels observed in patients with RA may in part help to explain the neutrophilia commonly associated with RA. The role of IL-6 in regulating neutrophil levels is further exemplified by studies demonstrating that decreased IL-6 signaling abrogated neutrophil count increases; it has been hypothesized that this result could be driven by a reversal in localization—ie, from the circulation to the marginated bone marrow pool.[58] This theory is markedly different from what is known about febrile neutropenia in myelosuppression, in which the cytotoxic effects of chemotherapy impact the production of neutrophils in the bone marrow.[59] Consistent with the bone marrow pool margination hypothesis, a decrease in IL-6 signaling has been associated with transient neutropenia, yet ex vivo experiments have not revealed any impact on pathogen-defense mechanisms (eg, phagocytosis and production of ROS).[57]

Neutrophil Migration

In addition to increased circulating levels of neutrophils, RA is characterized by a substantial increase in migration of neutrophils into the joint space.[55] The mechanisms that lead to the transmigration of neutrophils out of the circulation and into the tissues have been well studied.[13] Under conditions of inflammation, injury, or infection, mononuclear cells within the affected tissues become activated and release proinflammatory mediators such as granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-8, and IFN-γ.[61,62] These cytokines activate the vascular endothelium, upregulating the surface expression of adhesion molecules and inducing the production of chemokines, including IL-8.[13,55,63] The relatively high levels of these chemokines act as chemotactic gradients, attracting circulating neutrophils to the affected area.[64] Adhesion molecules expressed on the recruited neutrophils (eg, CD62L) interact with those on the activated endothelium, promoting margination followed by firm adhesion and transmigration out of the circulation.[11,55] Transmigrated neutrophils then home to sites of inflammation via chemotactic gradient, which can be established by bacterial products, such as *N*-formylmethionyl-leucyl-phenylalanine (fMLP) and LPS, complement components, or chemokines such as IL-8.[13,55] It is of interest that IL-8 is generated in high amounts in the joints of patients with RA by IL-6–activated FLS, and IL-6 has been found to enhance neutrophil migration toward IL-8 in vitro.[57,63] Furthermore, studies conducted both in vitro and in vivo have suggested that IL-6 may suppress neutrophil margination by reducing expression of CD162 on circulating neutrophils, in part by upregulating the production of IL-8.[65] In contrast, IL-6 was shown to enhance adhesion to endothelial cells in a dose-dependent manner in vitro, and suppression of IL-6 signaling reduced the migration of neutrophils into inflamed joints in animal models of collagen-induced arthritis (CIA).[66,67] While these data appear inconsistent with a role for IL-6 in suppressing neutrophil margination, other factors within the pathologic rheumatoid joint could compensate for the IL-6/IL-8–induced suppression of CD162, potentially maintaining a

local environment permissive for adhesion and transmigration. Indeed, in vitro experiments have shown that IL-6 suppression had no significant effect on the expression of several other neutrophil adhesion molecules, including CD11b, CD18, and L-selectin.[57]

Neutrophil Cytotoxicity

Neutrophils can cause extensive tissue damage due to the release of cytotoxic granules, which contain ROS, reactive nitrogen intermediates (RNI), proteolytic enzymes such as gelatinase and elastase, metalloproteinases, and other tissue-damaging molecules.[10,68,69] Degranulation can be induced by several triggers, including: bacterial products or viral proteins that are recognized by toll-like receptors (TLR); opsonized pathogens or other foreign particles that are recognized by complement receptors; IgG immune complexes that are recognized by Fcgamma receptors (FcγR); or by proinflammatory cytokines and chemokines that are recognized by their cognate receptors.[55,70,71] In healthy individuals, these interactions initiate a cascade of downstream events that include induction of the oxidative burst and subsequent release of low levels of cytotoxic molecules that help to contain and resolve the initiating stimulus (eg, infection).[68] In RA, however, excessive neutrophil degranulation is common, and the associated emission of large amounts of toxins can contribute to substantial tissue damage.[70]

Oxidative stress, initiated during degranulation and defined by a production of ROS in excess of the cell's neutralization capacity, can cause deleterious effects, such as injury to surrounding tissue and endothelial dysfunction (including increased vascular permeability, insulin resistance, and thrombosis), and can amplify the inflammatory cascade.[68,72,73] In RA, high levels of ROS have been detected in the synovial fluid of inflamed joints.[74] ROS also can initiate NET release and NETosis.[21] Cytokines can influence induction of oxidative stress, although the specific outcome may depend upon the stimulus. For example, TNF-α, GM-CSF, and IL-8 were each able to prime whole blood neutrophils to generate hydrogen peroxide in response to bacterial fMLP, while IL-1α, IL-1β, and IL-6 did not elicit an oxidative stress response.[75] In contrast, ROS production by FLS from RA synovial fluid was significantly upregulated by IL-6.[61] Recent studies have demonstrated that abrogation of IL-6 signaling may reduce the levels of oxidative stress markers in patients with RA relative to controls.[76]

In cases of chronic inflammation, the threshold for neutrophil activation may be lower than in healthy individuals, and indeed the neutrophil activation profiles in patients with RA are distinct from unaffected controls.[4] For example, circulating neutrophils from patients with RA exhibit an activated phenotype relative to healthy controls: they have higher chemotactic potential due to upregulated C-C chemokine receptor 2 (CCR2), enhanced phagocytic ability, and express higher levels of FcγR.[4,77,78] In RA, a key activation factor for neutrophils is the FcγR recognition of IgG-RF or IgM-RF immune complexes, which can be found in abundance as deposits within the synovial tissue and on the joint cartilage surface, or as soluble complexes in the synovial fluid.[79,80] FcγR-mediated endocytosis of soluble immune complexes can trigger neutrophil degranulation, NET formation, and NETosis.[81]

In addition, deposited immune complexes may induce "frustrated phagocytosis," or incomplete formation of phagocytic vesicles, which induces neutrophils to release high concentrations of proteases, ROS, and other cytotoxic agents directly onto the surface of the articular cartilage.[4,10]

IL-6 Regulation of Neutrophil-Mediated Inflammation

The elevated levels of IL-6 observed in the synovial fluid of patients with RA may cause an increased influx of neutrophils to the joint.[55] While the efflux of neutrophils from the vasculature is not generally considered a toxic process, rapid and intense infiltration of neutrophils into the synovium can cause acute injury and pathological inflammation.[11] Neutrophils and IL-6 also collaborate to maintain inflammation through establishment of positive feedback loops: activated neutrophils are a significant source of IL-6, which functions in an autocrine or paracrine fashion to further promote proinflammatory signaling **(Figure 3)**. Furthermore, neutrophils can affect IL-6 signaling by a process known as "shedding."[10] In this process, the membrane-bound IL-6 receptor (mIL-6R) is cleaved from the surface of the cell through the actions of the ADAM metalloproteinase domain 17 (ADAM17) to produce soluble IL-6R (sIL-6R).[8,82] IL-6 binds with similar affinity to both membrane and soluble forms of its receptor, and the membrane-bound or soluble cytokine-receptor complexes can each associate with membrane-bound glycoprotein 130 (gp130) to

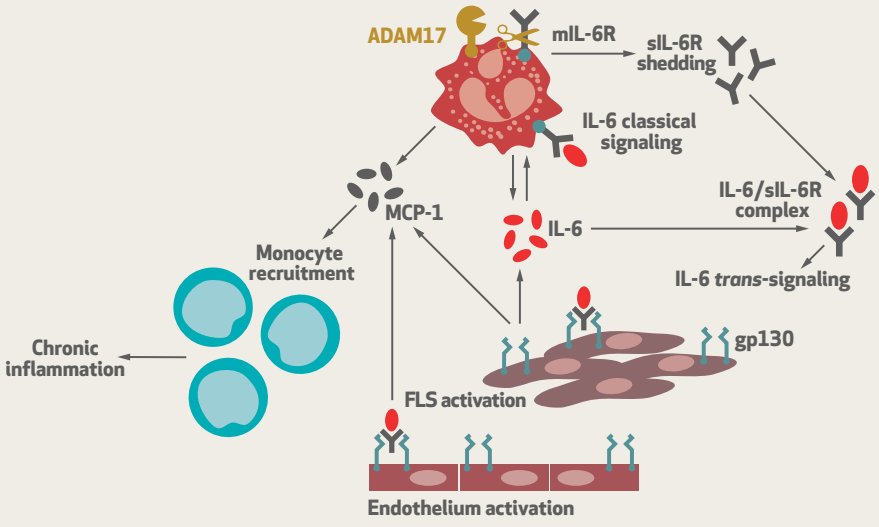

Figure 3. IL-6 signaling mechanisms initiate proinflammatory feedback loops in RA.[8,10,85,88] Activated neutrophils shed membrane-bound IL-6 receptor through the actions of metalloproteinases such as ADAM17, releasing a soluble form of the receptor that can bind IL-6 and signal in an IL-6 *trans*-signaling manner on cells expressing gp130, including FLS and endothelial cells. Activated FLS, in turn, release IL-6, which can further activate neutrophils and other immune cells via classical signaling and initiate a positive feedback loop of proinflammatory signaling. Activated neutrophils, FLS, and endothelial cells produce MCP-1, which recruits monocytes to the synovial space, initiating the transition from acute to chronic inflammation.

initiate intracellular signaling.[8,83,84] Classical, or *cis*, signaling transpires only in cells that express mIL-6 (primarily leukocytes and hepatocytes), while *trans*-signaling via IL-6/sIL-6R can occur in virtually any cell type expressing the ubiquitous gp130, including those that do not constitutively express mIL-6R.[8,83,85]

Initiation of Inflammatory Resolution

Under normal conditions, acute inflammation quickly comes under control as the clearance of the injurious stimuli completes its process within days, and invokes mechanisms to fully resolve the inflammation and any associated damage.[86] This process is achieved, in part, through IL-6 *trans*-signaling and neutrophil self-destruction. First, circulating neutrophils are recruited to sites of inflammation by factors secreted from activated endothelial cells, including IL-8.[85,87] IL-8 induces IL-6R shedding from the neutrophil surface, allowing for IL-6 *trans*-signaling on resident stromal cells such as FLS and endothelial cells.[7,87-89] The result is a downregulation of neutrophil-attracting chemokines (including IL-8) and augmentation of monocyte-attracting chemokines—in particular, monocyte chemoattractant protein-1 (MCP-1) and chemokine ligand 8 (CCL8)—abrogating further neutrophil accumulation and initiating monocyte recruitment. In healthy individuals, this shift in cell recruitment initiates inflammatory resolution: the accumulated neutrophils either age and undergo apoptosis, or undergo phagocytosis-induced cell death following clearance of the invading pathogens or other foreign particles.[85,88] Apoptotic neutrophils also shed IL-6R, further facilitating *trans*-signaling on proximal somatic cells and enhancing monocyte recruitment.[85] IL-6 *cis*-signaling upregulates macrophage colony-stimulating factor (M-CSF) on the surface of the recruited monocytes, promoting their differentiation into phagocytotic macrophages that ingest the apoptotic neutrophils, a process known as efferocytosis.[90,91] The efferocytosis process triggers macrophage production of anti-inflammatory mediators, such as TGF-β and IL-10, initiating downstream signaling cascades that effectively downregulate the inflammatory response and ultimately lead to resolution of inflammation.[92-94]

Removal of apoptotic neutrophils is critical in resolving the inflammatory process, and, in fact, any defects in the clearance of apoptotic cells and their associated debris often lead to induction or progression of inflammatory conditions.[10,94-96] In the case of inadequate or incomplete efferocytosis, apoptotic neutrophils undergo secondary necrosis, which is defined by a loss of membrane integrity and subsequent leakage of cellular contents, including inflammatory mediators, into the extracellular space.[91] Efferocytosis mechanisms appear to be largely intact in patients with established RA and NETs may also be efficiently resolved.[97] Studies on animal models have demonstrated that deficiencies in the clearance of apoptotic

debris can lead to a chronic polyarthritis that resembles human RA.[98,99] Studies also suggest that the expanded inflammatory effects of neutrophils in RA may be due to an abnormally extended life span due to dysregulation of apoptotic induction pathways, as discussed in the next section.

The Role of IL-6 in Neutrophil Apoptosis

As part of the normal neutrophil activation response, neutrophils receive antiapoptotic signals, which allow the innate immune response ample time to control an infection while an appropriate adaptive response is generated (usually 4 to 7 days); defects in the prosurvival pathways can cause susceptibility to certain infections.[100] Multiple lines of evidence suggest that in RA, both circulating and synovial neutrophils experience further delays in apoptosis, which may increase the likelihood of persistent inflammation and damage to host tissues and cells.[10,101] The role of cytokines in prolonging neutrophil survival has not been fully defined, but it has been shown that IL-6 can further upregulate the constitutively high levels of the anti-apoptotic protein Mcl-1 in RA-associated FLS. These levels are maintained by GM-CSF signaling, which itself is inducible by IL-6.[62,101-103] In addition, in ex vivo experiments using healthy human neutrophils, IL-6 induced the release of PAF which, in conjunction with IL-6 stimulation, has been shown to delay neutrophil apoptosis.[52] It has also been suggested that IL-6 is the exclusive proinflammatory signal whose function prolongs neutrophil survival in patients with osteomyelitis, a chronic bone infection.[104]

In vitro studies have suggested that the specific effects of IL-6 on neutrophil apoptosis may be dependent on microenvironment. The hypoxic environment of the synovium, for example, may promote neutrophil survival, as healthy neutrophils incubated with RA synovial fluid under hypoxic conditions exhibited an extended life span relative to those cultured in an oxygenated environment.[101] It is of interest that decreased IL-6 signaling was associated with reduced neutrophil survival and the oxidative and phagocytic activity of neutrophils when examined under hypoxic conditions, such as those seen in inflamed joints in RA.[105] The effects of IL-6 may be dependent upon concentration of neutrophils as well as activation status, which differ in pathological versus healthy states.[52,106-109] In fact, one study found that, in the presence of IL-6, a greater proportion of neutrophils survived when cultured more densely.[55,106] Indeed, IL-6 did not delay apoptosis of neutrophils cultured at concentrations that were representative of normal systemic human circulation.[106] Together, these data suggest that, in a state of chronic inflammation such as RA, IL-6 may support an abnormally and substantially extended life span of neutrophils, thereby allowing for an expanded contribution to articular inflammation and host tissue degradation.[63]

The Role of IL-6 and Neutrophils in the Transition From Acute to Chronic Inflammation in RA

The transition from acute to chronic inflammation in RA, which is characterized by a shift in the cellular composition of the synovium, may be in part directed by IL-6 *trans*-signaling.[1,85,88] In acute inflammation under normal conditions, the initial tissue infiltrate is primarily comprised of neutrophils; after approximately 1 to 2 days, however, monocytes predominate.[88] As discussed in a previous section, this shift in cellular composition is modulated by a shift in chemokine production from IL-8 to MCP-1 and, under normal conditions, resolution of inflammation is initiated.[85,88] In RA, the accumulation of long-lived neutrophils within the synovium, together with high levels of IL-6 and sIL-6R, may allow for sustained IL-6 *trans*-signaling and, as a result, increased monocyte recruitment.[88] In animal models of CIA and autoimmune arthritis (AIA), IL-6 was found to be central to monocyte recruitment, as IL-6$^{-/-}$ mice failed to develop the dense monocyte infiltrate that is characteristic of the models.[110,111]

Accumulation of monocytes within the synovium leads to formation of the pannus, a thickened and hypertrophic synovial lining. The pannus is composed of activated monocytes as well as macrophages, neutrophils, lymphocytes, and FLS, all of which secrete proinflammatory mediators.[60] These mediators contribute to the activation of osteoclasts, which reside at the interface between the pannus and the bone and are responsible for bone resorption, and FLS, which release soluble matrix metalloproteinases that degrade collagen in cartilage.[1,60,112] Pannus-associated neutrophils have also been shown to produce large amounts of proteolytic enzymes which invade and degrade the cartilage matrix and contribute to subchondral bone destruction in RA.[60,113] IL-6 may play a role in the sustained activation and invasiveness of all these cells by orchestrating feedback loops between them **(Figure 3)**. Studies of AIA and CIA in animal models suggest a critical role for IL-6 in pannus development and downstream tissue destruction. Two independent studies demonstrated that synovial hyperplasia was limited, pannus formation was abrogated, and no erosions of the articular cartilage and bone were noted in IL-6$^{-/-}$ mice.[110,111] Taken together, these results provide further evidence for the role of neutrophils in advancing the chronicity of RA, and of the contribution of aberrant IL-6 signaling to the pathologic effects of these cells.

Conclusions

Neutrophils are multifunctional leukocytes that serve as the first line of defense against invading pathogens and are critical mediators of inflammation-induced injury. In RA, these same functions may be amplified or dysregulated, contributing to a sustained and perpetuating pathogenic mechanism of autoantigen production, autoreactivity, and inflammation.[10] IL-6, a key proinflammatory cytokine, influences multiple neutrophil activities, beginning with their initial egress from the bone marrow and continuing to their death. In turn, neutrophils promote persistent IL-6 *cis*- and *trans*-signaling in inflamed tissues, ultimately driving the transition from acute to chronic inflammation.[85,88] Elevated IL-6 signaling can increase circulating neutrophil concentrations as well as their recruitment to sites of inflammation. IL-6 may also influence autoantigen generation, cytotoxic and NETotic capacity, and apoptotic timing.[1,52,56,57,85] Collectively, the interactions between neutrophils and the IL-6 signaling pathway contribute substantially to the pathogenic mechanisms that lead to chronic inflammation and tissue damage associated with RA.[55]

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SANOFI GENZYME

REGENERON